Supplemental material for

HEY1 Leu94Met gene polymorphism dramatically modifies its biological functions

by

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SUPPLEMENTARY MATERIALS AND METHODS

Plasmids

The following plasmids have been described: pSG5-HEY1, GST-HEY1, pSG5-HEY2, GST-AR-AF1-(1-556), GST-SRC1-PAS-(1-450), pSG5-SRC1e, pSG5-Gal4-DBD, pSG5-Gal4-HEY1, TAT-GRE-E1B-LUC, pGL2-Lex-Gal-Luc and pSG5-Lex-VP16 (Belandia *et al.*, 2005); p21-LUC, BAX-LUC, PIG3-LUC and pcDNA3-p53 (Llanos *et al.*, 2006); PIG3-del F (Contente *et al.*, 2002).

pSG5-HEY1-L94M, pSG5-HEY1-K51A/R53G/R51A, were generated by PCR site-directed mutagenesis. HEY1 region encoding the NLS (amino acids 46 to 58) was PCR-amplified and subcloned into pEGFP-C3 (Clontech, California, USA).

Quantitative real-time PCRs

Total RNA was extracted using TRI-reagent (Sigma-Aldrich) and treated with RNAse-free DNase (Invitrogen). Gene expression was analyzed by real-time RT-PCR using Brilliant SYBR-Green QPCR Master-Mix (Stratagene, California, USA). The human-specific primers used were: cyclin-B1-forward; AGAACCTGAGCCAGAACC, cyclin-B1-reverse; TTGCTCTTCCTCAAGTTGTC, cyclin-A2-forward; GAAGTACCAGACTACCATGAG, cyclin-A2-reverse; CTTCAAACTTTGAGGCTAACAG, Notch1-forward;

CAAGTTCTTGGTCCCTCCAG, Notch1-reverse; TGTGTTGCTGGAGCATCTTC, Jagged1-forward CGTAGTTCTACCGGCTTTGC, Jagged1-reverse; CAGTTCCAGCTTCACAGCAG, HES1-forward; CGGACATTCTGGAAATGACA, HES1-reverse; CATTGATCTGGGTCATGCAG RUNX2-forward; CAGACCAGCAGCACTCCATA, RUNX2-reverse; CAGCGTCAACACCATCATTC. p21-forward; CCTGTCACTGTCTTGTACCCT, p21-reverse; GCGTTTGGAGTGGTAGAAATCT, GADD45A-forward; ACGAGGACGACGACAGAGAT, GADD45A-reverse; GCAGGATCCTTCCATTGAGA, SKP2-forward; TGAGCTGCTCTTGGGAATCT, SKP2-reverse; AGCCAATGGTTGGTCCATAA, FOXM1-forward; GCTTGCCAGAGTCCTTTTTG, FOXM1-reverse; TCTCAAGCCTCCACCTGAGT, CENPA-forward; CTCCTGCACCCAGTGTTTCT, CENPA-reverse; CCTTTGGAACGGTGTTGATT, BIRC5-F; AGCCCTTTCTCAAGGACCAC, BIRC5-R; CAGCTCCTTGAAGCAGAAGAA, B99-forward; AGCCCCTAGTGAGGCTCTTC, B99-reverse; TCAGAGGCCTGCTTTCAGAT, MCG10-forward; TTTCTGGGTTATGGGAGCAG, MCG10-reverse; TGGGGACTCCAGGATAACAG, TOP2A-forward; GATCCTGCCAAAACCAAGAA, TOP2A-reverse; GTACAGATTTTGCCCGAGGA, CDC2-forward; CCATACCCATTGACTAACTATGGA, CDC2-reverse; GGAACCCCTTCCTCTTCACT, CDC25C-forward; GCTCAAGTCTTCGCCTGTGT, CDC25C-reverse; AGCCAGAGCTTCCTTCCTCT

The constitutively expressed L19 ribosomal gene was used as control to normalize mRNA expression (L19-forward; 5′-GCGGAAGGGTACAGCCAAT-3′, L19-reverse; GCAGCCGGCGCAAA).

Antibodies

The antibodies used were ANTI-FLAG M2 (Sigma-Aldrich, Missouri, USA), anti-p53 (DO-1, Santa Cruz Biotechnology, California, USA), anti-β-actin (AC-15, Sigma-Aldrich) anti-V5 (Invitrogen), MDM2 (SMP14, Santa Cruz Biotechnology, California, USA) and anti-p21 (C-19, Santa Cruz Biotechnology, California, USA).

SUPPLEMENTARY RESULTS

A human non-synonymous SNP converts HEY1 from an AR repressor to an AR activator

To test possible effects on HEY1 biological activity caused by the polymorphism L94M we generated the polymorphic variant by site-directed mutagenesis and tested the effect of L94M expression on the ability of AR to stimulate transcription from androgen-responsive luciferase reporter genes in transfected U2OS osteosarcoma cells. We found that the substitution L94M increased the activity of the AR-dependent reporter, transforming HEY1 from an AR repressor to a potent AR activator (Supplementary Figure 1a).

The SNP L94M does not affect the *in vitro* interaction between HEY1 and the androgen receptor or SRC1

HEY1 interacts directly with both the Activation Function-1 (AF1) domain in the androgen receptor and the Per-Arnt-Sim (PAS) domain in the nuclear receptor coactivator SRC1 through a complex interaction surface that requires several domains in HEY1, including the region containing the residue L94 (Belandia *et al.*, 2005). To explore the possibility that the L94M substitution affects protein-protein interactions between HEY1 and SRC1 or HEY1 and AR we fused the AR AF1 domain and the

SRC1 PAS domain respectively to GST and performed *in vitro* GST pull-down experiments. L94M showed a strong interaction with the SRC1 PAS domain and a weak but specific interaction with the AR AF1 domain (Supplementary Figure 1b), identical result to that observed for GST-HEY1 (Supplementary Figure 1b), suggesting that the change L94M does not affect the interaction between HEY1 and the AR or SRC1.

Characterization of U2OS-HEY1 and U2OS-L94M stable cell lines

Phase-contrast micrograph of monolayers of U2OS-HEY1 cells cultured for 11 days in the presence of tetracycline revealed that HEY1 expression induces striking changes in cell morphology indicating neuron-like differentiation (Supplementary Figure 2a). We then analyzed the effect of HEY1 expression on the expression of components of the Notch pathway (Notch1, Jagged1 and HES1) and RUNX2, a master regulator of osteoblast differentiation (Karsenty, 2008). We monitored mRNA levels by quantitative reverse transcription-PCR (QRT-PCR) and we observed that HEY1 expression, but not L94M expression, downregulated Jagged1 and RUNX2 (Supplementary Figure 2b). Expression of both HEY1 variants reduces HES1 and Notch1 mRNA levels, although the effects are more apparent upon expression of HEY1 induction (Supplementary Figure 2b). We also determined the effects of HEY1 and L94M on the expression of several cell cycle regulatory genes which expression or activity might be regulated directly or indirectly by p53 (reviewed in (Brown *et al.*, 2007; Riley *et al.*, 2008; Taylor and Stark, 2001)). HEY1 expression significantly inhibited mRNA levels for all but one (GADD45A) cell cycle regulatory genes examined. In contrast, L94M expression did not affect the expression of most genes analyzed. Only cyclin B1 and cyclin A2 were inhibited to similar levels upon L94M expression while p21 mRNA levels were moderately inhibited. Western blot analysis of

p21, a critical regulator of cell cycle, shown that expression of HEY1, but not of the polymorphic variant L94M, severely decreased endogenous p21 protein levels (Supplementary Figure 2d). These results indicate that HEY1 exerts profound effects in the expression of many genes involved in the control of cell differentiation and cell proliferation. Furthermore, the polymorphism L94M severely alters HEY1 effects on gene expression.

SUPPLEMENTARY REFERENCES

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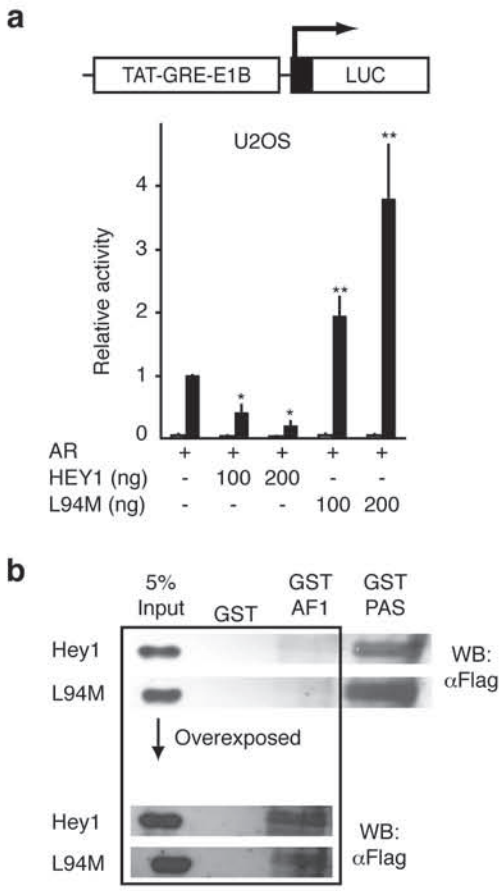
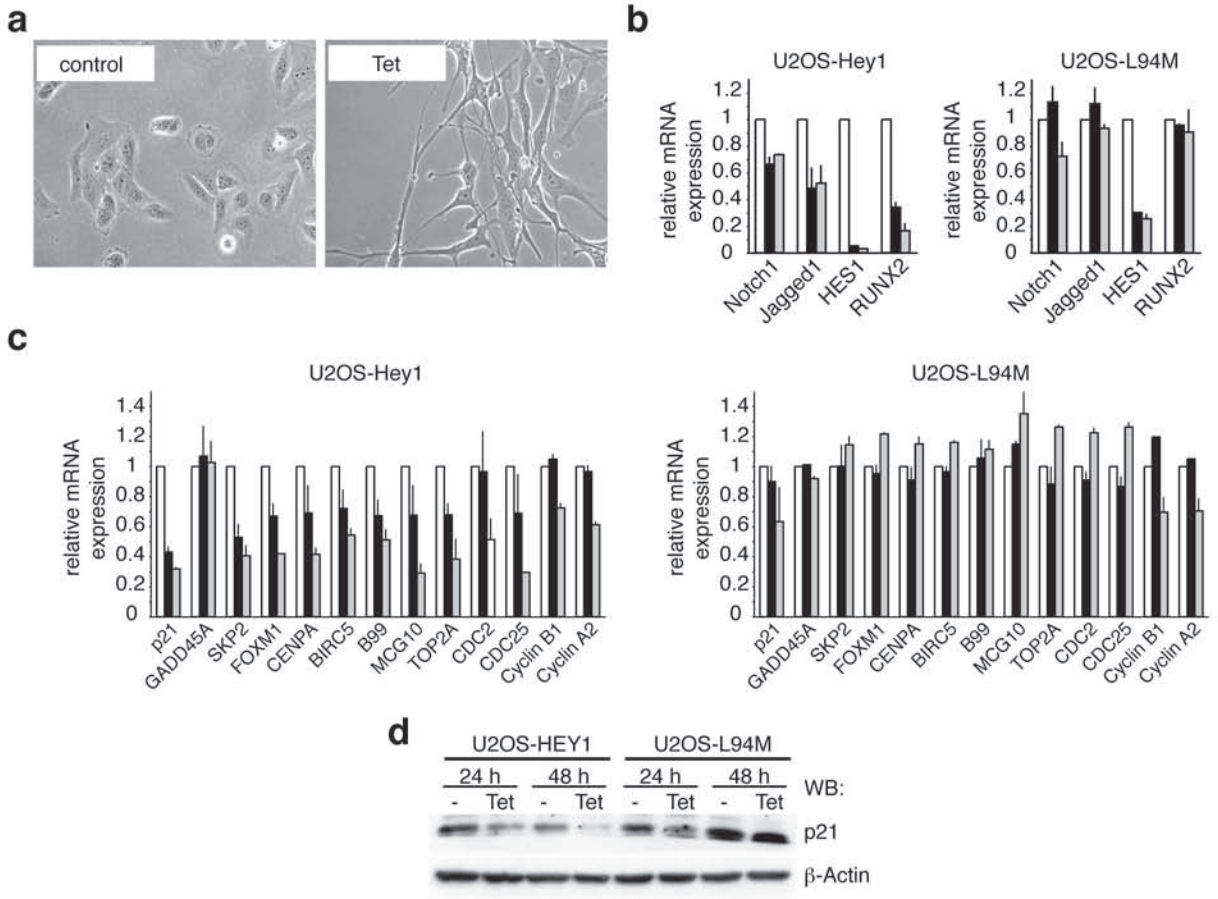
Supplementary Figures



Supplementary Figure 1 Characterization of the L94M variant. (**a**) L94M enhances AR-mediated hormone-dependent transactivation. U2OS cells were cotransfected with 100 ng of TAT-GRE-E1B-LUC, 25 ng of expression vector for AR and increasing amounts of expression vector for HEY1 or the variant L94M (100 or 200 ng). After transfection cells were washed and, when required, incubated 24 h in the presence of vehicle (white bars) or 10 nM DHT (black bars). Subsequently, cell lysates were assayed using a dual luciferase reporter system. Normalized values are expressed relative to the activity of AR alone in the presence of DHT. The results shown represent the averages of results of at least three independent experiments assayed in duplicate + SD. *, by student´s t test, P was <0.001 compared with values for controls. **, by student´s t test, P was <0.005 compared with values for controls. (**b**) The L94M variant interacts in vitro with SRC1 and AR. Whole cell extracts from COS-1 cells previously transfected with expression vectors for Flag-tagged HEY1 or the variant L94M were incubated with GST fusion proteins containing the SRC1 bHLH PAS domain or the AR AF1 domain coupled with Sepharose beads. The associated HEY1 was detected by western blotting using anti-Flag tag antibody.



Supplementary Figure 2 Characterization of U2OS-HEY1 and U2OS-L94M stable cell lines. (**a**) Phase-contrast micrograph of monolayers of U2OS-HEY1 cells cultured for 11 days in the presence (Tet) or in the absence (control) of 1 μg/ml tetracycline. (**b**) Quantitative RT-PCR analysis for the expression of Notch1, Jagged1, HES1 and RUNX2 transcripts in U2OS-HEY1 and U2OS-L94M cells cultured in the absence (white bars) or in the presence of 1 μg/ml tetracycline for 24 h (black bars) or 48 h (gray bars). The results shown represent the averages of results of two independent experiments assayed in duplicate + SD. (**c**) Quantitative RT-PCR analysis for the expression of cell cycle regulatory gene transcripts in U2OS-HEY1 and U2OS-L94M cells cultured in the absence (white bars) or in the presence of 1 μg/ml tetracycline for 24 h (black bars) or 48 h (gray bars). The results shown represent the averages of results of two independent experiments assayed in duplicate + SD.(**d**) Immunoblot analysis of p21 and β-actin in U2OS-HEY1 cells treated with 1 μg/ml tetracycline (Tet) for 24 h or 48 h.