(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
27 December 2007 (27.12.2007)

PCT

(10) International Publication Number
WO 2007/149550 A2

(51) International Patent Classification:
G01N 33/53 (2006.01)

(21) International Application Number:
PCT/US2007/014537

(22) International Filing Date: 22 June 2007 (22.06.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/816,053 22 June 2006 (22.06.2006) US

(71) Applicant *(for all designated States except US)*: **THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK** [US/US]; 412 Low Memorial Library, 535 West 116th Street, New York, NY 10027 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **ACCILI, Domenico** [IT/US]; 541 W. 113th Street, #4c, New York, NY 10025 (US). **KITAMURA, Tadahiro** [JP/US]; 1422 Oleary Terrace, Fort Lee, NJ 07024 (US). **FUNAHASHI, Yashuhiro** [JP/US]; 235 West 48th Street, #19J, New York, NY 10036 (US). **KITAJEWSKI, Jan, K.** [US/US]; 323 Hillcrest Road, Ridgewood, NJ 07450 (US).

(74) Agents: **LOVE, Jane, M.** et al.; Wilmer Cutler Pickering Hale And Dorr LLP, 399 Park Avenue, New York, NY 10022 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2007/149550 A2

(54) Title: MODULATION OF DIFFERENTIATION AND CELL FUNCTION VIA FOX01 AND NOTCH SIGNALING

(57) Abstract: Methods of identifying compounds useful for regulating differentiation via interactions with FoxOl are provided.

MODULATION OF DIFFERENTIATION AND CELL FUNCTION VIA FOXO1 AND NOTCH SIGNALING

[0001] All patents, patent applications and publications cited herein are hereby incorporated by reference in their entirety. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

[0002] This patent disclosure contains material that is subject to copyright protection. The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or the patent disclosure as it appears in the U.S. Patent and Trademark Office patent file or records, but otherwise reserves any and all copyright rights

TECHNICAL FIELD

[0003] This application relates to the field of cell signaling.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0004] The U.S. Government may have certain rights in this invention pursuant to Grant No. RO1 HL62454 awarded by National Institutes of Health.

BACKGROUND

[0005] Forkhead01A (FOX01A, forkhead in rhabdomyosarcoma, forkhead box 01A, FKHR, FKH1) is a member of the O sub-family of forkhead (Fox) proteins that regulate hormone-induced differentiation. FoxO proteins are subject to post-translational modifications including phosphorylation and acetylation. FoxO1 phosphorylation regulates myoblast and pre-adipocyte differentiation, and gain-of-function mutations in FoxO1 prevent both processes.

[0006] The Notch pathway plays a role in differentiation, e.g., neural, vascular, muscular, and endocrine differentiation, during embryogenesis. Upon ligand-induced cleavage, the intracellular domain of the Notch receptor (Notch-IC) translocates to the nucleus, where it interacts with the DNA binding protein Csl (which stands for CBF1, Suppressor of Hairless, and Lag-1; also termed Rbp-Jκ) changing the transcriptional properties of Notch from a suppressor to an activator of transcription. Csl targets include

the *Hairy and Enhancer of Split* (Hes) genes. Hes1 controls gut endoderm, preadipocyte, and myoblast differentiation. Myogenic differentiation is regulated via Hes1 inhibition of the myogenic effector MyoD.

[0007] FoxO and Notch signal through two seemingly distinct mechanisms; FoxO via the phosphatidylinositol-3-kinase pathway, and Notch via the Hes pathway. Expression of FoxO1A has been reported to be of importance in some alveolar rhabdosarcomas, type II diabetes, cancer, muscular dystrophy, autoimmunity, and premature ovarian failure.

SUMMARY

[0008] The invention relates to the finding that Fox01and Notch cooperate in the regulation of differentiation. FoxO1 regulates the Notch signaling pathway by direct interaction with the Notch target Csl to activate Hes1 transcription. Accordingly, the invention relates to a method for identifying a compound capable of modulating Notch activity. The method includes contacting a FoxO1 polypeptide or fragment of a FoxO1 polypeptide with a Csl polypeptide or fragment of a Csl polypeptide under protein binding conditions, and optionally, admixing a test compound with the FoxO1 polypeptide and the Csl polypeptide, and determining whether the test compound inhibits the binding of FoxO1 polypeptide with Csl polypeptide when compared to the binding in the absence of the compound, such that a test compound that can inhibit the binding is a compound capable of modulating Notch activity. In some embodiments, the test compound is admixed with one or more of the polypeptides before contacting, during contacting, after contacting, or any combination thereof. A fragment of a FoxO1 polypeptide can, in some embodiments, bind a Csl polypeptide (e.g., a Csl polypeptide that is between about 10 amino acids to about 500 amino acids, or a Csl polypeptide that is at least about 500 amino acids in length, for example, a Csl polypeptide or fragment that includes amino acids 179-272 of a full-length Csl protein. In certain embodiments of the method, the FoxO1 polypeptide includes from about 1 to about 300 contiguous amino acids from the amino terminus of a FoxO1 sequence. The FoxO1 polypeptide can include, in some cases, from about 20 to about 300 contiguous amino acids from the amino terminus of a FoxO1 sequence.

[0009] The method can also include determining one or more of the following; whether the test compound modulates Hes1 expression in a cell, or whether the compound modulates expression of at least one of: an angiogenesis gene, a myogenesis gene, a

neurogenesis gene, a gut development gene, an adipogenic gene, or a pancreatic β cell differentiation gene.

[0010] In some embodiments of the method, the contacting occurs in a cell, e.g., a myoblast, a pre-adipocyte, a neuronal precursor cell, a PC-12 cell, a gut endoderm cell, an embryonic stem cell, or a pancreatic duct cell, or a derivative of any of the foregoing cell types.

[0011] Another aspect of the invention includes a method for identifying a compound capable of inhibiting binding of a FoxO1 protein and a Csl protein. The method includes admixing a compound with a FoxO1 polypeptide or a fragment thereof and a Csl polypeptide under protein-binding conditions, and measuring the amount of FoxO1 polypeptide bound to Csl, such that a decrease in the amount of FoxO1 bound to Csl in the presence of the compound compared to the amount of FoxO1 bound in the absence of the compound indicates that the compound is capable of inhibiting the interaction between Csl and FoxO1. In certain embodiments, the FoxO1 is a fragment of FoxO1 that can bind to Csl. In some cases, the method includes determining one or more of: whether the test compound modulates Notch activity; whether the test compound modulates *Hes1* expression in a cell; or whether the compound modulates expression of at least one of: an angiogenesis gene, a myogenesis gene, a neurogenesis gene, a gut development gene, an adipogenic gene, a pancreatic β cell differentiation gene, or any combination thereof. Yet other embodiments include determining whether the compound inhibits binding of the Csl protein to the N-terminal region of the FoxO1 protein, whether the compound binds to the N-terminal region of the FoxO1 protein whether the test compound binds to the Csl protein, determining whether the test compound binds to all or a portion of amino acids 1-300 of a Csl protein, determining whether the test compound binds to all or a portion of amino acids 179-272 of a human Csl or a homolog thereof. In certain embodiments of the methods described herein, the cell is a myoblast, a pre-adipocyte, a neuronal precursor cell, a PC-12 cell, a pancreatic β cell precursor, a gut endoderm cell, an embryonic stem cell, or a pancreatic duct cell, or a derivative of any of the foregoing cell types.

[0012] Another aspect of the invention is a compound capable of inhibiting an interaction between FoxO1 and Csl comprising a peptide from about amino acid position 1 to about amino acid position 300 of a Fox01 protein, an antibody, an antibody fragment, a peptide, a peptoid, a non-nucleic acid small organic molecule, or other small molecule. In

certain embodiments, the peptide is derived from a protein sequence represented by Genbank accession no. NM_019739.

[0013] Yet another aspect of the invention is a method for inhibiting muscle differentiation. The method includes contacting a myoblast with a compound that can enhance the interaction between FoxO1 and Csl. In certain embodiments, the myoblast is in a mammal, e.g., a human. In another embodiment, the compound includes one or more of a FoxO1 polypeptide, a Csl-binding fragment of FoxO1 polypeptide, a nucleic acid encoding a FoxO1 polypeptide or a fragment thereof, a Csl polypeptide, a FoxO1-binding fragment of a Csl polypeptide, a nucleic acid encoding a Csl polypeptide or fragment thereof, or any combination thereof.

[0014] The invention also relates to a method for increasing muscle differentiation. The method includes contacting a myoblast with a compound that can inhibit the interaction between FoxO1 and Csl. In some cases, the myoblast is in a mammal, e.g., a human.

[0015] The invention also relates to a method of modulating differentiation. The method includes contacting an undifferentiated cell with a compound that modulates the interaction between Csl and FoxO1. In some cases, the compound inhibits the interaction between FoxO1 and Csl and differentiation is enhanced. In certain embodiments, the compound enhances myoblast differentiation, adipogenesis, angiogenesis, pancreatic β cell differentiation, or neurite sprouting. In yet another embodiment of the invention, the compound enhances the interaction between FoxO1 and Csl and differentiation is decreased. In certain embodiments of the method, the compound inhibits myoblast differentiation, angiogenesis, pancreatic β cell differentiation, or neurite sprouting.

[0016] Another aspect of the invention is a method of inhibiting expression of *Hes1*. The method includes contacting a cell that can express *Hes1* with a compound that inhibits FoxO1 protein expression or activity. In certain embodiments, the compound includes an siRNA or interfering RNA (RNAi) that specifically inhibits expression of a FoxO1 gene.

DESCRIPTION OF THE DRAWINGS

[0017] FIG. 1A is a reproduction of a set of micrographs of C_2C_{12} cells transduced with adenoviruses as indicated and immunostained with anti-myosin and DAPI (4', 6-diamidino-20-phenylinole).

[0018] FIG. 1B is a reproduction of a micrograph of C_2C_{12} cells transduced with HA-FoxO1-ADA or HA-Notch1-1C adenovirus and immunostained with anti-HA antibody and DAPI.

[0019] FIG. 1C is a reproduction of a Western blot of C_2C_{12} cells transduced with a sequence encoding FoxO1-ADA, Notch1-1C, or Notch decoy and assayed for myosin expression.

[0020] FIG. 1D is a reproduction of a Western blot of C_2C_{12} cells co-transfected with FoxO1 siRNA and a sequence encoding FLAG-FoxO1 or Green Fluorescent Protein (GFP). The ability of Foxo1 siRNA to inhibit expression of endogenous (left panel) and transfected (right panel) Foxo1 following adenoviral transduction was tested.

[0021] FIG. 1E is a reproduction of a semiquantitiave RT-PCR analysis of *Myf5*, *MyoD*, and myosin expression during embryogenesis.

[0022] FIG. 1F is a reproduction of RT-PCR analyses of *MyoD* and *yf5* in $Fox1^{+/+}$ embryos at E8.5 and E9.5 (n=6 for each genotype). An asterisk indicates $P<0.01$ by ANOVA (Analysis Of Variance).

[0023] FIG. 1G is a reproduction of a photomicrograph of C_2C_{12} cells transduced with HA-FoxO1-ADA or HA-Notch1-IC adenovirus and stained with anti-HA antibody and DAPI.

[0024] FIG. 1H is a reproduction of a Western blot of FoxO1, FoxO3, and FoxO4 expression in C_2C_{12} cells transfected with FoxO1 siRNA.

[0025] FIG. 1I is a reproduction of a photomicrograph of C_2C_{12} cells transduced with lacZ, FoxO1-ADA, or Notch1-IC adenovirus, stained with anti-Ki67 antibody and DAPI. The numbers indicate the Ki67 labeling index as a percentage of Ki67-positive cells (at least 1,000 cells counted.)

[0026] FIG. 1J is a reproduction of a Western blot of FoxO1-ADA and siRNA-resistant FoxO1-ADA in cells transfected with FoxO1 siRNA.

[0027] FIG. 1K represents a bar graph of a morphometric analysis of Myosin-positive cells. Results from differentiation experiments were analyzed by scoring the number of Myosin-immunostained cells as percentage of all DAPI-positive cells.

[0028] FIG. 1L depicts DBD-Foxo1ADA reporter gene assays that were carried out using the canonical Foxo1-responsive *Igfbp1* promoter (left panel) and the *Hes1* promoter (right panel) in cells co-transfected with Foxo1-ADA or DBD-Foxo1ADA. Western blot (inset) demonstrates that expression levels of the two proteins are similar. An asterisk indicates $P<0.01$ by ANOVA.

[0029] FIG. 2A is a reproduction of the result of a co-immunoprecipitation experiment in which endogenous FoxO1 and Csl expressing C_2C_{12} cells were co-cultured with LacZ- (-) or Jagged1-expressing HEK293 cells (+). "IP" (immunoprecipitation) indicates the antigen against which the precipitating antibody was directed.

[0030] FIG. 2B is a reproduction of the result of a co-immunoprecipitation experiment in C_2C_{12} cells. IP is immunoprecipitation, IB is immunoblotting, and TCL is total cellular lysate.

[0031] FIG. 2C is a reproduction of the result of a co-immunoprecipitation experiment using C_2C_{12} cells.

[0032] FIG. 2D is a reproduction of the result of an experiment in which cells were co-transfected with FLAG-Csl, HA-FoxO1, or HA-Notch1-IC.

[0033] FIG. 2E is a reproduction of the result of an experiment in which cells were co-transfected with FLAG-Csl, HA-FoxO1, or HA-Notch1-IC.

[0034] FIG. 2F is a reproduction of the result of a co-immunoprecipitation experiment in which C_2C_{12} cells were co-transfected with FLAG-Csl and the truncated mutant Myc- or HA-tagged Δ256 FoxO1.

[0035] FIG. 2G is a reproduction of the result of a co-immunoprecipitation experiment in which C_2C_{12} cells were co-transfected with FLAG-Csl and the truncated mutant Myc- or HA-tagged Δ256 FoxO1.

[0036] FIG. 2H is a reproduction of the result of an immunoprecipitation experiment

[0037] FIG. 2I is a reproduction of the immunoblot result of an experiment in which full-length and truncated fragments of GST-FoxO1 and GST-FLAG/Csl were purified from bacteria, co-incubated, and Csl isolated using an anti-FLAG antibody. Immunoprecipitates were detected with anti-FoxO1 or anti-FLAG antibodies.

[0038] FIG. 2J is a reproduction of the immunoblot result of an experiment in which full-length and truncated fragments of GST-FoxO1 and GST-FLAG/Csl were purified from bacteria, co-incubated, and Csl isolated using an anti-FLAG antibody. Immunoprecipitates were detected with anti-FoxO1 or anti-FLAG antibodies

[0039] FIG. 2K is a reproduction of an experiment in which a *Hes1* promoter ChIP spanning the Csl binding site in C_2C_{12} cells was used to detect endogenous FoxO1, Csl, and Notch 1 (Endog.); or following transduction with FoxO1-ADA (FoxO10ADA during myoblast differentiation. Input represents DNA extracted from chromatin prior to immunoprecipitation. *Hes1* expression (semiquantitative RT-PCR) and myosin expression (Western blot) corresponding to each time point are shown. Day 0 is the time when cells are serum-deprived to induce myoblast fusion.

[0040] FIG. 2L is a reproduction of the results of experiments in which cells were co-transfected with FoxO3, FoxO4 expression vectors and a Csl expression vector, immunoprecipitated with antibody specific to a FoxO, and the immunoprecipitates assayed for co-immunoprecipitation of the specific FoxO1 and Csl.

[0041] FIG. 2M is a bar graph depicting the results of assays of reporter gene activity using promoter assays of murine *Csl* promoter (-1536 to 22) following co-transfection of C_2C_{12} cells with wild-type FoxO1 or FoxO1-ADA.

[0042] FIG. 3A is a bar graph depicting the results of *Hes1* reporter gene assays in HEK293 cells transduced with FoxO1-ADA, Notch 1-IC, FoxO1, siRNA GFP siRNA, or control plasmid. Luciferase activity was assayed and normalized to β-galactosidase activity. The data are represented by arbitrary units relative to control empty vector.

[0043] FIG. 3B is a reproduction of the result of an experiment in which *Hes1, Hes5,* and *Hey1* expression was measured by semiquantitative RT-PCR in C_2C_{12} cells transduced with FoxO1-ADA or Notch1-IC following transfection of GFP, FoxO1, or Csl siRNA as indicated.

[0044] FIG. 3C is a bar graph depicting the results of assays of reporter gene activity using promoter assays of a synthetic *Hes1* reporter gene containing four tandem repeats of a Csl binding site and co-transfection with FoxO1 and Notch1-IC in C_2C_{12} cells.

[0045] FIG. 3D is a pair of bar graphs depicting the results of assays of reporter gene activity using the FoxO1-responsive Igfbp1 promoter (left graph) and the *Hes1* (right graph) in cells co-transfected with FoxO1-ADA or DBD-FoxO1ADA. The inset is a reproduction of a Western blot detecting expression levels of FoxO1-ADA and DBD-FoxO1ADA. An asterisk indicates that $P<0.01$ by ANOVA.

[0046] FIG. 4A is a reproduction of the results of a ChIP assay of endogenous FoxO1 and Notch1 in C_2C_{12} cells co-cultured with LacZ- (-) or Jagged1-expressing HEK293 cells (+) in the absence (lanes 1-2) and presence (lanes 3-4) of Csl siRNA.

[0047] FIG. 4B is a reproduction of the results of ChIP assays of endogenous Notch1 in a co-culture system in the absence (lanes 1-2) and presence (lanes 3-4) of FoxO1 siRNA.

[0048] FIG. 4C is a bar graph depicting the results of Hes1 promoter assays following co-culture in the absence or presence of FoxO1 GFP siRNA.

[0049] FIG. 4D is a reproduction of the results of ChIP assays of NcoR, SMRT, and MAML1 binding to *Hes1* in the co-culture system in the absence (lanes 1-2) and presence (lanes 3-4) of FoxO1 siRNA.

[0050] FIG. 4E is a reproduction of the results of RT-PCR detecting expression of *MyoD*, *Myf5*, and *β-actin* in C_2C_{12} cells.

[0051] FIG. 4F is a drawing depicting a model of FoxO1 and Notch regulation of the Hes1 promoter.

[0052] FIG. 4G is a reproduction of a Western blot depicting the Csl levels in C_2C_{12} cells following transfection with Csl siRNA at various concentrations.

[0053] FIG. 5A is a reproduction of a set of micrographs of PC12 cells transduced with FoxO1-ADA, Notch1-IC, or Notch decoy using adenoviruses in the presence or absence of FoxO1 siRNA as indicated.

[0054] FIG. 5B is a reproduction of a set of micrographs of 3T3-F442A preadipocytes transduced with FoxO1-ADA, Notch1-IC, or Notch decoy using adenoviruses and in the presence or absence of FoxO1 siRNA as indicated. Triglyceride accumulation in 3T3-F442A was assayed using oil red-O staining.

[0055] FIG. 5C is a reproduction of a set of micrographs of HUVECs transduced with FoxO1-ADA, Notch1-IC, or Notch decoy using retroviruses in the presence or absence of FoxO1 siRNA as indicated. PSI is the presenilin (γ-secretase) inhibitor compound E (5 μM).

[0056] FIG. 5D is a bar graph depicting the percentage of PC12 cells transduced with FoxO1 and Notch expression constructs, and induced with nerve growth factor (NGF) that exhibited neurite outgrowth. An asterisk indicates $P<0.01$ by ANOVA.

[0057] FIG. 5E is a bar graph depicting the percentage of oil red O-positive 3T3-F442 cells in cultures transduced with FoxO1 and Notch expression constructs. An asterisk indicates $P<0.01$ by ANOVA.

[0058] FIG. 5F is a bar graph depicting the percentage of HUVECs transduced with FoxO1 and Notch expression constructs that exhibited tube formation. An asterisk indicates $P<0.01$ by ANOVA.

[0059] FIG. 6A is a reproduction of results from a Western blot where Foxo1 and Foxo4 expression levels were analyzed.

[0060] FIG. 6B is a reproduction of metachromatic and immunohistochemical micrographs depicting soleus and plantaris muscle from Myog-Foxo1 mice and control (lox/lox) littermates.

[0061] FIG. 6C are bar graphs representing gene expression analysis of Myog-Foxo1 (sold bars) and control mice (empty bars). Data are means ± SEM of three independent measurements (n= 6 for each genotype). An asterisk indicates $P<0.05$ by ANOVA. TropC: troponin-C; TropT: troponin-T; Mlc: myosin light chain; Myog: Myogenin; Mck: muscle-type creatine kinase.

[0062] FIG. 6D are bar graphs depicting a treadmill performance test in 8 week-old Myog-*Foxo1* mice and *lox/lox* littermates (n=6 for each genotype). An asterisk indicates $P<0.05$ by ANOVA.

DETAILED DESCRIPTION

[0063] This patent disclosure contains material that is subject to copyright protection. The copyright owner has no objection to the facsimile reproduction by anyone of the patent document or the patent disclosure as it appears in the U.S. Patent and Trademark Office patent file or records, but otherwise reserves any and all copyright rights.

[0064] All patent applications, published patent applications, issued and granted patents, texts, and literature references cited in this specification are hereby incorporated herein by reference in their entirety to more fully describe the state of the art to which the present invention pertains.

[0065] As various changes can be made in the methods and compositions described herein without departing from the scope and spirit of the invention as described, it is intended that all subject matter contained in this application and claims, shown in the accompanying drawings, or defined in the appended claims be interpreted as illustrative, and not in a limiting sense.

[0066] Other features and advantages of the invention will be apparent from the detailed description, drawings, and from the claims.

[0067] FoxO transcription factors convey anti-differentiation signals that can be removed via Akt-dependent phosphorylation. Notch signaling plays a role in cell fate determination in multiple lineages, including myoblasts, endothelial cells, and pre-adipocytes. It has been found that FoxO1 regulates the Notch pathway by engaging the Notch target Csl to activate hes1 transcription. Constitutively active FoxO1 can inhibit differentiation and such inhibition can be at least partially reversed with a Notch antagonist. In addition, suppression of FoxO1 expression prevents Notch inhibition by decreasing Notch binding to Csl and thus decreasing expression of Hes1 and its target genes. It has also been found that the effect of FoxO1 on transcription is mediated via a protein-protein interaction, not a protein-DNA interaction.

Screening Assays

[0068] The invention provides methods (also referred to herein as "screening assays") for identifying modulators, i.e., candidate compounds (e.g., proteins, peptides, peptidomimetics, peptoids, small non-nucleic acid small organic molecules, small inorganic molecules, heteroorganic molecules, organometallic molecules, nucleic acids (e.g., oligonucleotides, siRNA, and nucleic acids containing non-naturally occurring nucleic acids) or other drugs) that can modulate the binding of a FoxO1 protein and a Csl protein. Compounds thus identified can be used to modulate the activity of FoxO1 and Csl, for example resulting in modulation of Notch activity. For example, a compound that interferes with the interaction between a FoxO1 protein and a Csl protein can inhibit one or more activities associated with Notch, e.g., the differentiation process is inhibited by Notch-IC.

[0069] In certain methods of the invention, nucleic acid sequences or amino acid sequences are used, e.g., in a screening assay. Appropriate sequences, e.g., FoxO1 sequences, Notch sequences, and Csl sequences are known in the art. Known sequences (e.g., from a human) are generally used although one in the art will know how to identify suitable homologs of useful sequences that are also suitable for use. An example of a suitable human Csl sequence is GenBank accession no. NM_015874. A suitable human FoxO1 sequence is GenBank accession no. NM_019739.

[0070] In one embodiment, the invention provides assays for screening test compounds to identify compounds that bind to a FoxO1 polypeptide or a Csl polypeptide. Such compounds can then be tested for their ability to increase or decrease the interaction between a FoxO1 polypeptide and a Csl polypeptide.

[0071] In another embodiment, an assay is conducted to identify compounds that promote or inhibit the interaction between a FoxO1 and a Csl polypeptide.

[0072] In one embodiment, an assay is a cell-based assay in which a cell that expresses a FoxO1 protein or biologically active portion thereof and a Csl protein or biologically active portion thereof is contacted with a test compound, and the ability of the test compound to modulate Notch activity is determined. Determining the ability of the test compound to modulate Notch activity can be accomplished by monitoring, for example, the activity of Hes1 or by monitoring the ability of the cell to differentiate in the presence and absence of the test compound. Alternatively, the efficacy of a test compound can be

determined by measuring the binding of proteins known as transcriptional coactivators and corepressors to the Notch target, Hes1. In the case of a differentiation assay, induction or expression or activity of one or more markers associated with differentiation of the specific cell type can be assayed, cell morphology can be assayed (e.g., sprouting by a PC-12 cell), or a function of the cell can be monitored. The cell, for example, can be from an invertebrate, a non-human mammal such as a mouse, rat, rabbit, sheep, cow, horse, pig, or non-human primate, or a human.

[0073] Markers of differentiation that are useful in the assays described herein include, but are not limited to, myogenin, myosin, glucose transporter 4, leptin, peroxisome proliferation-activating receptor gamma (PPARγ), and myelin basic protein.

[0074] The ability of the test compound to modulate binding of a FoxO1 polypeptide and a Csl polypeptide can also be evaluated. Pairs of FoxO1 and Csl polypeptides that can bind to each other are referred to as binding partners. An assay can be performed, for example, by coupling one of the polypeptides with a label (e.g., a radioisotope or enzymatic label) such that binding of the labeled polypeptide with the partner can be determined by detecting the labeled compound in a complex. The binding partners are incubated in the presence and absence (control) of a test compound, the bound material separated from the unbound material, and the amount of labeled binding partner associated with the unlabeled binding partner is assayed. In some cases, the amount of labeled binding partner that is unbound is assayed. In either case, the amount of binding in the presence of the test compound is compared to the amount of binding in the absence of the test compound. A difference in the amount of binding between the binding partners in the presence of the test compound compared to the amount of binding in the absence of the test compound indicates that the test compound can modulate the interaction between the binding partners. For example, a test compound that can decrease the binding between a FoxO1 and a Csl polypeptide is a candidate compound for promoting differentiation, e.g., in a cell for which differentiation is inhibited by Notch.

[0075] Methods known in the art can be used for generating a detectable label. For example, a polypeptide can be labeled with a radiolabel such as ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting or detection of radioemission or by scintillation counting. Alternatively, a polypeptide can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or

luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product. In some cases, a polypeptide is synthesized as a heterologous polypeptide that includes a detectable label such as a FLAG peptide or a green fluorescent protein (GFP).

[0076] The ability of a test compound to modulate the interaction between the binding partners can be performed without the labeling of any of the interactants. For example, the ability of a test compound to modulate the interaction of a FoxO1 polypeptide and a Csl polypeptide can be detected, e.g., using a microphysiometer, without the labeling of either the compound or the FoxO1 polypeptide (McConnell et al., 1992, Science 257:1906-1912. As used herein, a "microphysiometer" (e.g., Cytosensor) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between the binding partners in the presence and absence of the test compound.

[0077] In general, cell-free assays involve preparing and incubating a reaction mixture of a FoxO1 polypeptide and a Csl polypeptide under conditions and for a time sufficient to allow the two components to interact and bind, thus forming a complex that can be removed and/or detected. A test compound is incubated with one of the binding partners prior to, during, or after the binding partners interact, and the amount of interaction in the presence and absence of the test compound is assayed. A test compound that can alter the interaction (e.g., the amount of binding, Kd, or Ka) is a compound that can modulate the effects of FoxO1 on Notch signaling. In general, FoxO1 and Csl are used in approximately equimolar concentrations.

[0078] In another embodiment, a cell-free assay is provided in which a FoxO1 protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to bind to the FoxO1 protein or biologically active portion thereof is evaluated. Biologically active portions of a FoxO1 protein to be used in an assay of the present invention include fragments that participate in interactions with a Csl molecule. Once a compound that can bind to the FoxO1 is identified, it is generally tested for its ability to affect at least one of; the activity of a FoxO1 or the ability to modulate the interaction between a FoxO1 and a Csl.

[0079] The interaction between two molecules can also be detected, e.g., using fluorescence resonance energy transfer (FRET)(for example, Lakowicz et al., U.S. Patent No. 5,631,169; Stavrianopoulos et al., U.S. Patent No. 4,868,103; and fretimaging.org/mcnamaraintro.html). A fluorophore label on the first, 'donor' molecule is selected such that the emitted fluorescent energy of the donor is absorbed by a fluorescent label on a second, 'acceptor' molecule, which in turn fluoresces due to the absorbed energy. Alternately, the 'donor' protein molecule can utilize the natural fluorescent energy of tryptophan residues. Labels are chosen that emit different wavelengths of light, such that the 'acceptor' molecule label can be differentiated from that of the 'donor'. Since the efficiency of energy transfer between the labels is related to the distance separating the molecules, the spatial relationship between the molecules can be assessed. In a situation in which binding occurs between the molecules, the fluorescent emission of the 'acceptor' molecule label in the assay should be maximal. An FET binding event can be conveniently measured through standard fluorometric detection means well known in the art (e.g., using a fluorimeter).

[0080] In another embodiment, determining the ability of binding partners to interact in the presence and absence of a test compound, or the ability of a test compound to bind to one of the binding partners can be accomplished using real-time Biomolecular Interaction Analysis (BIA) (e.g., Sjolander and Urbaniczky, 1991, Anal. Chem. 63:2338-2345 and Szabo et al., 1995, Curr. Opin. Struct. Biol. 5:699-705). "Surface plasmon resonance" or "BIA" detects biospecific interactions in real time, without labeling any of the interactants (e.g., BIAcore). Changes in the mass at the binding surface (indicative of a binding event) result in alterations of the refractive index of light near the surface (the optical phenomenon of surface plasmon resonance (SPR)), resulting in a detectable signal that can be used as an indication of real-time reactions between biological molecules.

[0081] In some assays, one of the binding partners is anchored onto a solid phase. The binding partner/test compound complexes anchored on the solid phase can be detected after a binding reaction. In general, the binding protein can be anchored onto a solid surface, and the test compound (which is not anchored) can be labeled, either directly or indirectly, with detectable labels discussed herein. The binding partner to be immobilized can be anchored directly to a solid phase (e.g., a particle, bead, plate, slide, or other suitable surface), or can be indirectly immobilized, for example, via an antibody that recognizes the binding partner

and is attached to the solid phase. Formats in which one of the binding partners is immobilized are useful to facilitate separation of complexed from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. In general, an antibody used in such an assay specifically recognizes one of the binding partners, but does not interfere with binding of the binding partners to each other. Such antibodies can be derivatized to the wells of the plate, and the binding partner trapped in the wells by antibody conjugation.

[0082] Binding of a test compound to a binding partner, or the interaction of binding partners in the presence and absence of a test compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtiter plates, test tubes, slides, chips, and micro-centrifuge tubes. Suitable modifications can be made to one or both of the binding partners to facilitate an assay using a particular vessel or surface. For example, a fusion protein can be provided that adds a domain to a binding partner that allows binding to a matrix. For example, a glutathione-S-transferase/FoxO1 fusion protein or a glutathione-S-transferase/Csl fusion protein can be adsorbed onto glutathione Sepharose® beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtiter plates, which are then combined with the test compound or the test compound and the non-adsorbed binding partner, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Following incubation, the beads or microtiter plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, an the amount of complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of interaction between the binding partners determined using techniques known in the art.

[0083] Other techniques for immobilizing a binding partner on a matrix include using conjugation of biotin and streptavidin. One of the binding partners is biotinylated, for example, using biotin-NHS (N-hydroxy-succinimide) using techniques known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemicals).

[0084] To conduct the assay, the non-immobilized component is added to the surface containing the anchored component and the two components are incubated for sufficient time and under conditions the permit binding. After the incubation is complete, unreacted

components are removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished using methods known in the art. When the initially non-immobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the initially non-immobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the initially non-immobilized component (the antibody, in turn, can be directly labeled or indirectly labeled with, e.g., a labeled anti-Ig antibody). The amount of complex formed in the presence and absence of a test compound can be determined to identify test compounds that increase binding between the binding partners, or to identify compounds that decrease binding between the binding partners.

[0085] Cell free assays can be conducted in a liquid phase. In such an assay, the reaction products are separated from unreacted components by any of a number of techniques, including but not limited to differential centrifugation (for example, Rivas et al., 1993, Trends Biochem. Sci. 18:284-7), chromatography (gel filtration chromatography, ion-exchange chromatography), electrophoresis (e.g., Ausubel et al., eds. Current Protocols in Molecular Biology, 1999, J. Wiley: New York.), and immunoprecipitation (for example, Ausubel et al., eds., 1999, Current Protocols in Molecular Biology, J. Wiley: New York). Such resins and chromatographic techniques are known to those in the art (e.g., Heegaard, 1998, J. Mol. Recognit. 11:141-8; Hage, 1997, J. Chromatogr. B. Biomed. Sci. Appl. 699:499-525). Further, fluorescence energy transfer may also be conveniently utilized, as described herein, to detect binding without further purification of the complex from solution.

[0086] In another embodiment, the assay includes contacting a FoxO1 or Csl binding partner or biologically active portion thereof with a known compound that binds to the binding partner to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with the binding partner, wherein determining the ability of the test compound to interact with the binding partner is determined by assaying the ability of the test compound to preferentially bind to the binding partner or biologically active portion thereof as compared to the known compound.

[0087] FoxO1 stabilizes the Notch-IC/Csl complex. Accordingly, assays can be performed that determine the ability of a test compound to dissociate FoxO1 from a Notch-IC/Csl complex. Alternatively, a test compound can be assayed for the ability to prevent a FoxO1 polypeptide from binding to such a complex.

[0088] The invention provides methods for determining the ability of the test compound to modulate the activity of a FoxO1 polypeptide through modulation of the expression or activity of a downstream gene or gene product such as Hes1. In some cases, the ability of a test compound to inhibit or promote an activity regulated by a FoxO1/Csl interaction is assayed. Examples of such assays include assays in which a myoblast is contacted with a test compound and the effect of the test compound on Myogenesis is analyzed. In general, this type of assay is used after identification of a candidate compound that can bind FoxO1 or a candidate compound that can modulate the interaction between a FoxO1 polypeptide and a Csl polypeptide.

[0089] In some cases employing the methods described herein, it is not necessary to perform a control with every test reaction and the results of a test reaction are compared to a pre-established standard. Controls or comparisons to pre-established standards are together referred to as a "reference."

[0090] As described herein, assays can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring one of the binding partners onto a solid phase, and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the binding partners, e.g., by competition, can be identified by conducting the reaction in the presence of the test substance. Alternatively, test compounds that disrupt preformed complexes, e.g., compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. Examples of the various formats are briefly described below.

[0091] In a heterogeneous assay system, either of the binding partners is anchored onto a solid surface (e.g., a microtiter plate), while the non-anchored species is labeled, either

directly or indirectly. The anchored species can be immobilized by non-covalent or covalent attachments. Alternatively, an immobilized antibody specific for the species to be anchored can be used to anchor the species to the solid surface.

[0092] In an alternate embodiment of the invention, a homogeneous assay can be used. For example, a preformed complex of the binding partners is prepared in which one of the binding partners is labeled, but the signal generated by the label is quenched due to complex formation (see, e.g., U.S. Patent No. 4,109,496). The addition of a test substance that competes with and displaces one of the species from the preformed complex will result in the generation of a signal above background. In this way, test substances that disrupt binding partner interaction can be identified.

[0093] In yet another aspect, FoxO1 and Csl polypeptides can be used as "bait proteins" in a two-hybrid assay or three-hybrid assay (see, e.g., U.S. Patent No. 5,283,317; Zervos et al., 1993, Cell 72:223-232; Madura et al., 1993, J. Biol. Chem. 268:12046-12054; Bartel et al., 1993, Biotechniques 14:920-924; Iwabuchi et al., 1993, Oncogene 8:1693-1696; Licitra et al., 1996, Proc. Nat. Acad. Sci. USA 93:12817-21, and Brent WO94/10300), to identify polypeptide test compounds, that bind to or interact with one or both of the binding partners to modulate their interaction. Such test polypeptides can be activators or inhibitors of signaling affected by the interaction between FoxO1 and Csl, e.g., Notch signaling.

Identification of Compounds

[0094] Other methods of identifying compounds are known in the art (e.g., Best et al., 2004, Proc. Nat. Acad. Sci. 101:17622-27), and the skilled practitioner can apply such methods using information provided herein.

[0095] In another aspect, the invention pertains to a combination of two or more of the assays described herein. For example, a compound that can modulate the interaction between a FoxO1 polypeptide and a Csl polypeptide can be identified using a cell-based or a cell free assay, and the ability of the agent to modulate the activity of Notch signaling can be confirmed *in vivo*, e.g., in an animal model suitable for examining Notch-pathway differentiation or a disorder related to a Notch signaling pathway.

[0096] This invention further pertains to novel agents identified by the screening assays described herein. Accordingly, it is within the scope of this invention to further use a

modulating agent identified as described herein (e.g., an agent that can increase the interaction or activity of a FoxO1/Csl complex or an agent that can inhibit the interaction or activity of a FoxO1/Csl complex) in an appropriate animal model to determine the efficacy, toxicity, side effects, or mechanism of action, of treatment with such an agent. Furthermore, novel agents identified by the above-described screening assays can be used for treatments as described herein.

Animal Models

[0097] Candidate compounds that affect the interaction between FoxO1 and Csl can be tested for their ability to modulate disorders related to the interaction between FoxO1 and Csl (e.g., disorders that may be ameliorated by interfering with the interaction between FoxO1 and Csl). Those in the art will know of animal models suitable for such testing. Such animal models include, without limitation, animal models of diabetes (e.g., Accili et al., 1996, Nat. Genet. 12:106-109), muscular dystrophy (e.g., Guo, 2005, Dan. Med. Bull. 52:117), neurodegenerative disorders such as Parkinson's disease (e.g., Gonzales et al., 2006, Brain Res. Jan 11, epub.; Fleming et al., 2005, NeuroRx, 2:495-503), Huntington's disease, multiple sclerosis (e.g., Owens, 2006, Adv. Neurol. 98:77-89; 't Hart et al., 2006, Drug. Disc. Today, 11:58-66), and trauma of the spinal cord.

Candidate compounds are tested by administering various dosages of the compound that the animal model and determine whether features of the targeted disease are modulated. Tested features can include, for example, changes in RNA expression, changes in protein expression, changes in all metabolism or structural features, or changes disease symptoms demonstrated by the animal.

Test Compounds

[0098] The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone that are resistant to enzymatic degradation but that nevertheless remain bioactive; see, e.g., Zuckermann et al. (1994, J. Med. Chem. 37:2678-85); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library

and peptoid library approaches are limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, 1997 Anticancer Drug Des. 12:145).

[0099] Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al. (1993, Proc. Natl. Acad. Sci. USA. 90:6909), Erb et al. (1994, Proc. Natl. Acad. Sci. USA 91:11422), Zuckermann et al. (1994, J. Med. Chem. 37:2678), Cho et al. (1993, Science 261:1303), Carrell et al. (1994, Angew. Chem. Int. Ed. Engl. 33:2059), Carell et al. (1994, Angew. Chem. Int. Ed. Engl. 33:2061), and in Gallop et al. (1994, J. Med. Chem. 7:1233).

[0100] Libraries of compounds can be presented in solution (e.g., Houghten, 1992, Biotechniques 13:412-421), or on beads (Lam, 1991, Nature 354:82-84), chips (Fodor, 1993, Nature 364:555-556), bacteria (Ladner, U.S. Patent No. 5,223,409), spores (Ladner U.S. Patent No. 5,223,409), plasmids (Cull et al., 1992, Proc. Natl. Acad. Sci. USA 89:1865-1869), on phage (Scott and Smith (1990, Science 249:386-390; Devlin, 1990, Science 249:404-406; Cwirla et al., 1990, Proc. Natl. Acad. Sci. 87:6378-6382; Felici, 1991, J. Mol. Biol. 222:301-310; Ladner *supra.*), or on other suitable substrates.

[0101] In some embodiments, the compound can interact with Csl and is derived from a naturally occurring Csl binding protein other than a Csl polypeptide or a FoxO1 polypeptide. Examples of naturally occurring Csl binding proteins include Kaposi's sarcoma-associated herpes virus RTA protein, Epstein-Barr virus proteins (the EBNA-2 and -3 proteins of Epstein-Barr Virus (EBV) (Grossman et al. 1994; Hsieh and Hayward 1995; Johannsen et al. 1996)) and the 13S isoform of adenovirus E1A (Ansieau et al. 2001).

[0102] Toxicity and therapeutic efficacy of compounds identified as described herein can be determined using known pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit high therapeutic indices are generally used. While compounds that exhibit toxic side effects may be used, care should be taken to design a

delivery system that targets such compounds to the site of affected tissue to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[0103] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds generally lies within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Methods of Treatment

[0104] Compounds that can modulate the interaction between FoxO1 and Csl are useful for modulating differentiation of various cell types.

[0105] In one embodiment, a myoblast is treated by contacting the myoblast with a compound that that decreases the expression or activity of FoxO1. In response to such treatment, the myoblast can initiate differentiation. Such treatments are useful for, e.g., muscle regeneration in damaged tissues, for use in conjunction with muscle transplants, and in myodegenerative diseases such as muscular dystrophy, in which it is desirable to stimulate differentiation or in conjunction with gene therapy methods in which muscle progenitor cells are transplanted into a subject and stimulated using a compound as described herein to promote muscle differentiation.

[0106] Compounds that can inhibit the interaction between FoxO1 and Csl are also useful for treatment of disorders in which it is desirable to promote one or more features of neurogenesis, e.g., neurite sprouting. Such disorders include nerve damage or neurodegenerative disorders, including, without limitation, neurodegenerative disorders such as Parkinson's disease, Huntington's disease, multiple sclerosis, and trauma of the spinal cord. In other embodiments, a compound that enhances the interaction between FoxO1 and Csl is useful for treating a disorder in which it is desirable to inhibit one or more

features of neuronal development such as sprouting. For example, such compounds may be useful *in vitro* to drive differentiation of neural progenitors into specific neuronal sub-types, e.g., for use in the preparation of neuronal cells for therapy of neuromuscular disorders and spinal cord injury.

[0107] Compounds that affect FoxO1/Csl interaction, for example, as described herein, are useful for treatment of disorders associated with FoxO1 and Csl. For example, such compounds can be employed to treat diabetes by one or more of the following; reducing FoxO activity at the promoters of genes controlling liver glucose production and liver lipid synthesis, storage, and release; increasing the differentiation of endocrine stem cells into insulin-producing beta cells in the pancreas; decreasing the output of neuropeptides that influence appetite and satiety from the hypothalamus; treating vascular complications of diabetes by way of compound-eluting stents applied to arteries of diabetic individuals (for example, during angioplasty); decreasing the number or size of fat cells and thus decreasing body fat content. Compounds that affect the interaction between FoxO and Csl can also be useful to prevent or slow the growth of Notch-dependent cancers. For example, at least a subset of the compounds identified as described herein can be Notch antagonists and thus can be used to inhibit cellular dedifferentiation in cancers associated with Notch activity, e.g., breast or prostate cancer.

Pharmaceutical Compositions

[0108] Compounds identified as described herein (also referred to herein as "active compounds") can be incorporated into pharmaceutical compositions. Such compositions typically include the active compound and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" includes solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Supplementary active compounds can also be incorporated into the compositions.

[0109] A pharmaceutical composition is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, inhalation, transdermal (topical), transmucosal, and rectal administration; or oral administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile

diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[0110] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the selected particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In some cases, one or more isotonic agents are included, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be effected by including in the composition one or more agents that delay absorption, for example, aluminum monostearate and gelatin.

[0111] Sterile injectable solutions can be prepared by incorporating the active compound in the specified amount in an appropriate solvent with one or a combination of ingredients enumerated above, as needed, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and other ingredients selected from those enumerated

above or others known in the art. In the case of sterile powders for the preparation of sterile injectable solutions, the methods of preparation are known in the art and include, for example, vacuum drying and freeze-drying, which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0112] Oral compositions generally include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules, e.g., gelatin capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

[0113] For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser that contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

[0114] Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

[0115] The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

[0116] In one embodiment, an active compound is prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release

formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations are known to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

[0117] It is advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the selected pharmaceutical carrier.

[0118] As defined herein, a therapeutically effective amount of an active compound (i.e., an effective dosage) that is a protein or peptide ranges from about 0.001 to about 30 mg/kg body weight, about 0.01 to about 25 mg/kg body weight, about 0.1 to about 20 mg/kg body weight, about 1 to about 10 mg/kg body weight, about 2 to about 9 mg/kg body weight, about 3 to about 8 mg/kg body weight, about 4 to about 7 mg/kg body weight, or about 5 to about 6 mg/kg body weight. The active compound can be administered one time per week for between about 1 to about 10 weeks, between about 2 to about 8 weeks, between about 3 to about 7 weeks, or for about 4 weeks, about 5 weeks, or about 6 weeks. The skilled artisan will appreciate that certain factors may influence the dosage and timing to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or antibody can include a single treatment or can include a series of treatments.

[0119] For antibodies or antibody fragments, the dosage is generally about 0.1 mg/kg of body weight (generally about 10 mg/kg to about 20 mg/kg). If the antibody is to act in the brain, a dosage of about 50 mg/kg to about 100 mg/kg is usually appropriate. Generally, partially human antibodies and fully human antibodies have a longer half-life within the

human body than other antibodies. Accordingly, lower dosages and less frequent administration is often possible. Modifications such as lipidation can be used to stabilize antibodies and to enhance uptake and tissue penetration (e.g., into the brain). A method for lipidation of antibodies is described by Cruikshank et al. (1997, J. Acquired Immune Deficiency Syndromes and Human Retrovirology 14:193).

[0120] The present invention encompasses agents that can, for example, be small molecules. For example, such small molecules include, but are not limited to, peptides, peptidomimetics (e.g., peptoids), amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, non-nucleic acid organic compounds, or inorganic compounds (i.e., including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

[0121] Exemplary doses include milligram or microgram amounts of the small molecule per kilogram of subject or sample weight (e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram. It is furthermore understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. When one or more of these small molecules is to be administered to an animal (e.g., a human) to modulate expression or activity of a polypeptide or nucleic acid of the invention, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

[0122] An active agent that is a nucleic acid molecule can be inserted into a vector and used as gene therapy vectors. An active agent that is a protein or peptide can also be

provided to a subject by inserting a nucleic acid sequence encoding the protein or polypeptide into a vector, which is used as a gene therapy vector. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see e.g., Chen et al., 1994, Proc. Natl. Acad. Sci. USA 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is embedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can include one or more cells that produce the gene delivery system.

[0123] The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

[0124] Without committing to any particular theory, it appears that Notch/FoxO cooperation integrates environmental cues through Notch with metabolic cues through FoxO1 to regulate progenitor cell maintenance and differentiation. This provides a general mechanism for committed progenitor cells to avoid differentiation in response to developmental cues transmitted by Notch signaling when FoxO1 is active (i.e., in the absence of growth factors). Such cells can persist in a dormant state in adult tissues in which they can undergo terminal differentiation in response to a combination of Notch ligand and hormonal/nutritional cues that lead to FoxO1 inhibition. For example, diabetes is associated with excessive angiogenesis in the eye and in the coronary arteries. In the former case, the angiogenesis leads to blindness; in the latter, to plaque rupture and infarcts. By inhibiting the FoxO1Notch interaction, vascular differentiation is slowed and the incidence of such serious complications of diabetes can be decreased. In the pancreas, Notch signaling has been implicated in regulating differentiation of beta cells from endocrine stem cells. In the pancreas, it can be beneficial at certain stages of the disease to inhibit Notch to promote beta cell differentiation, which can result in more insulin secretion and improved glucose control. There is also evidence that cancer arises from stem cells. In such cases, regulation of the FoxO/Notch interaction can be used to prevent cancer stem cells from dividing and expanding. Also, FoxO is an effector of the signaling pathway mediated by PTEN, the most commonly mutated oncogene in human cancer. Thus, modulation of a FoxO signaling pathway, as described herein, is useful for inhibition of cancer progression.

EXAMPLES

[0125] The invention is further illustrated by the following examples. The examples are provided for illustrative purposes only. They are not to be construed as limiting the scope or content of the invention in any way.

[0126] Foxo1 gain-of-function (3-5) phenocopies Notch1 activation (13, 17, 22, 23) in every cellular differentiation context. Moreover, Foxo1 ablation (24) phenocopies Notch1 ablation (25) in mice. Despite these intriguing similarities, Foxo and Notch signal through two seemingly distinct mechanisms, the phosphatidylinositol-3-kinase pathway (Foxo), and the Hes/Hey pathway (Notch). In this study, we show that Foxo physically and functionally interacts with Notch by promoting co-repressor clearance from Csl, thus controlling the myogenic program.

[0127] Myogenic precursors arise from mesodermal stem cells (26) and are converted into myotubes by a multi-step process culminating in the expression of myogenic transcription factors of the MRF family (MyoD, Myogenin, MRF4 and Myf5) (27). Myogenic transcription factors heterodimerize with E proteins and promote expression of muscle-specific genes, acting in close coordination with myocyte-specific MEF2 enhancer factors (28).

[0128] Adult muscle is a heterogeneous tissue, primarily defined by its myofiber content (29). Different myosin heavy chain (MyHC) sub-types characterize different myofibers. Type I fibers express primarily slow-twitch MyHC, whereas type II fibers express fast-twitch MyHC (29). The process of fiber-type specification is controlled at multiple steps. First, there appears to be heterogeneity among myogenic precursor cells, and evidence from avian embryo cross-transplantation experiments indicates that early precursors contribute primarily to slow muscle fibers, and later precursors to fast fibers (29). Post-natally, fiber type specification is also affected by cell autonomous factors, including innervation and endocrine/nutritional cues (28). The Foxo co-activator Pgc1α plays a critical role in promoting the formation of slow-twitch fibers (30), and recent data have also implicated the Foxo deacetylase Sirt1 in this process (31). Using conditional mutagenesis in mice, we show that Foxo1's role in suppressing MyoD-dependent myogenesis in C2C12 cells is mirrored by an increase of MyoD-containing myofibers in

Foxo1-deficient skeletal muscle, consistent with a key function in myoblast lineage specification.

[0129] Foxo transcription factors govern metabolism and cellular differentiation. Unlike Foxo-dependent metabolic pathways and target genes, the mechanisms by which these proteins regulate differentiation have not been explored. Activation of Notch signaling mimics the effects of Foxo gain-of-function on cellular differentiation. Using muscle differentiation as a model system, we show that Foxo physically and functionally interacts with Notch by promoting co-repressor clearance from the Notch effector Csl, leading to activation of Notch target genes. Inhibition of myoblast differentiation by constitutively active Foxo1 is partly rescued by inhibition of Notch signaling, while Foxo1 loss-of-function precludes Notch inhibition of myogenesis and increases MyoD expression. Accordingly, conditional Foxo1 ablation in skeletal muscle results in increased formation of MyoD-containing (fast-twitch) muscle fibers and altered fiber type distribution at the expense of Myogenin-containing (slow-twitch) fibers. Notch/Foxo1 cooperation may integrate environmental cues through Notch with metabolic cues through Foxo1 to regulate progenitor cell maintenance and differentiation.

Example 1: Materials and Methods

Animal generation and analysis

[0130] Myogenin-cre (49) and *Foxo1*^{flox} mice have been described (9). The wild-type, null and *Foxo1*^{flox} alleles were detected using PCR with primers 5'-GCT TAG AGC AGA GAT GTT CTC ACA TT-3', 5'-CCA GAG TCT TTG TAT CAG GCA AAT AA-3' and 5'-CAA GTC CAT TAA TTC AGC ACA TTG A-3'. Prior to the treadmill performance test, mice were trained for 2 days (Columbus Instruments). The test was performed at 15 m/min for the first 30 min, followed by 1m/min increases at 10 min intervals until exhaustion. Skeletal muscle samples were quickly frozen in OCT matrix, and 7 μm serial sections were obtained. Muscle fibers were typed using metachromatic ATPase (50) or immunostaining with anti-skeletal slow myosin (Sigma). For embryonic studies, we set up timed matings of heterozygous Foxo1 (24) or Notch1 (25) mice and recovered embryos at E9.5. mRNA was isolated from whole embryos and real-time RT-PCR was performed as described below

Adenovirus, retrovirus and expression vectors

[0131] FoxO1-ADA, Notch1-IC, Jagged 1, Csl, and Notch-decoy adenoviral and mammalian expression vectors were as described in Nakae et al. (2001, J. Clin. Invest. 108:1359-1367) and Das et al. (2004, J. Biol. Chem. 279:30771-30780). Retroviruses expressing FoxO1-ADA and Notch1-IC were generated using the pQCXIH vector (BD Biosciences/Clontech, Palo Alto, CA). To generate a Notch decoy (pAdlox Notch 1ECD-Fc), the extracellular domain of Notch 1(bp 241-4229, GenBank Accession No. X57405) was fused in-frame with a human IgG Fc tag and cloned into pAdlox (Clontech). Retroviral supernatant was produced from cells transiently co-transfected with pVSV-G (BD Biosciences/Clontech) vector and designated pQCXIH vector into GP2-293 cells (BD Biosciences, Palo Alto, CA). FoxO1 and GFP siRNAs were from Santa Cruz Biotechnology, Inc. To generate the DNA binding-deficient Foxo1, N208 and H212 were replaced with alanine and arginine, respectively, using QuikChange Mutagenesis Kit (Stratagene). The mutations were then cloned in the backbone of the Foxo1-ADA mutant.

Cell differentiation

[0132] C_2C_{12} cells (a murine myoblast cell line) and 3T2-F442A cells (a pre-adipocyte cell line) were differentiated as described in Nakae et al. (2003, Dev. Cell 4:119-129) and Hribal et al. (2003, J. Cell Biol. 162:535-541). PC12 cells (American Type Culture Collection; ATCC) were differentiated with medium containing 1% horse serum and 100 ng/ml nerve growth factor (NGF). Neurite outgrowth was assayed by counting cells possessing neurites longer than two cell bodies. Human umbilical vein endothelial cells (HUVECs) were isolated, cultured, and differentiated using the EGM-2 Bullet kit (CAMBREX; Das et al., 2004, J. Biol. Chem. 279:30771-80). After retroviral transduction, hygromycin B-resistant clones were generated. Tube formation assays were performed as described in Funahashi et al. (2002, Cancer Res. 62:6116-6123).

mRNA studies

[0133] Messenger RNA isolation, northern blotting, semiquantitative reverse trancriptase-polymerase chain reaction (RT-PCR), and real-time RT-PCR were performed using techniques known in the art. Primer sequences were designed and tested using methods known in the art.

[0134] Messenger RNA from embryos (Hosaka et al., 2004, Proc. Natl. Acad. Sci. USA 101:2975-2980) was obtained from timed pregnancies at embryonic days (E) 8.5 through 11.5 and used for real time RT-PCR analyses.

Luciferase assay and co-culture assay

[0135] HEK293 cells were transfected with murine Hes1- reporter sequence (base pairs -194 to 160 relative to the transcription start of murine Hes1 (HES1/pGL2 basic). Synthetic Hes1- reporter sequence (containing a four Csl binding sites operatively linked to the Hes1-reporter sequence, 4x Csl/pGL2 basic) or Csl-luciferase (-1536 to 22 Csl/pGL2 basic) reporter sequence along with pCMV5, pCMV5-FoxO1-ADA, pQNC-Notch1-IC, pHyTc Notch decoy or FoxO1 SiRNA. Plasmid pRSW-β-galactosidase was used as a control for transfection efficiency (Das et al., 2004, J. Biol. Chem. 279:30771-80). For co-culture assays, Notch 1 was expressed in C_2C_{12} cells and Jagged1 or LacZ in HEK293 cells by transfection. HEK293 cells were harvested and seeded on C_2C_{12} cells. After incubation for one hour, the co-cultured cells were used in experiments.

Western blotting and immunoprecipitation

[0136] Western blotting and immunoprecipitation assays were performed using techniques known in the art with anti-myosin (MF-20), anti-hemagglutinin (anti-HA; 12CA5, Boehringer Mannheim, Indianapolis, IN), anti-FLAG (M2, Sigma, St. Louis, MO), anti-FoxO1 (H128 and N20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Notch 1 (C-20, Santa Cruz Biotechnologies, Inc.) anti-Csl (Chemicon International, Inc.; Temecula, CA and Santa-Cruz Biotechnology, Inc.), anti-NcoR (Santa-Cruz Biotechnology, Inc.), anti-SMRT (anti-silencing mediator for retinoid and thyroid receptors; Santa-Cruz Biotechnology, Inc.) or anti-MAML1 (anti-Mastermind-like; Chemicon International, Inc.) antibodies. For FoxO/Csl co-immunoprecipitation, purified nuclear fractions were used. Because Csl migrates close to IgG heavy chain on SDS-PAGE, dimethylpyrimilidate (DMP from Pierce Biotechnology, Inc., Rockford, IL) was used to cross-link antibodies to Protein A beads and avoid IgG contamination of eluted protein complexes (Chi et al., 2004, Methods Enzymol. 377:299-316).

Chromatin immunoprecipitation assays (ChIP)

[0137] ChIP assay in C_2C_{12} cells was performed as described in Nakae et al. (2003, Dev. Cell 4:119-129) and in co-cultured cells as described by Fryer et al. (2004, Mol. Cell 16:509-520). The primer pairs employed to amplify the Csl binding site of the Hes1 promoter were 5'-GCA AAG CCC AGA GGA AAG AGT TAG-3' and 5'-AGG AGA GAG GTA GAC AGG GGA TTC-3'.

siRNA transfection and siRNA-resistant FoxO1

[0138] For experiments using siRNA, the FoxO1-specific siRNA sequence was AC GGA GGA TTG AAC CAG TAT A. The Csl specific siRNA sequence was TAG GGA AGC TAT GCG AAA TTA. siRNA was transfected using Lipofectamine™-Plus reagent (Invitrogen Corp., Carlsbad, WA). siRNA-resistant FoxO1 was generated by replacing three residues (underlined) in the sequence AC GGC GGTCTG AAC CAG TAT A.

DNA binding deficient (DBD) FoxO1

[0139] To generate BD FoxO1, N208 and H212 of FoxO1 were replaced to be alanine and arginine residues, respectively using a QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA). The mutations were then introduced in the backbone of the FoxO1-ADA mutant.

Recombinant proteins and interaction assays

[0140] GST-FLAG-Csl encompassing amino acids 1-527, 1-279, 1-172 and 279-527 fragments were generated by cloning into pGEX6P-1. GST-FoxO1 constructs have been described (Puigserver et al., 2003, Nature 423:550-555). Following bacterial culture and IPTG induction, GST fusion proteins were purified and incubated together. Thereafter, GST-FLAG/Csl was isolated by immunoprecipitation with anti-FLAG antibody, the immune pellets were washed extensively, and immunoblots were performed using anti-FoxO1 antiserum.

Example 2: Interaction of FoxO1 and Notch Signaling in Myoblast Differentiation

[0141] To test whether the FoxO1 and Notch pathways interact, the ability of FoxO1 and Notch to regulate myoblast differentiation was examined. C2C12 cells undergo myogenic conversion and myotube fusion upon growth factor withdrawal, a process

associated with Foxo1 nuclear translocation (3). In C_2C_{12} cells, transduction of adenovirus encoding a constitutively active (always in the nucleus, as opposed to endogenous FoxO that shuttles to and from the nucleus) FoxO1 mutant (FoxO1-ADA) prevented myoblast fusion induced by serum withdrawal. Constitutive expression of FoxO1 was also associated with greater than 80% decrease in myosin expression (FIG 1A, FIG. 1B, FIG. 1G). Conversely, FoxO1 inhibition by siRNA did not affect these processes (FIG. 1A and 1B). The FoxO1 siRNA effectively suppressed expression of both transfected FLAG-FoxO1 (FIG. 1C) and endogenous FoxO1 (FIG. 1D) in a dose-dependent manner, without affecting control proteins or other FoxO isoforms (FIG. 1H). Myoblast differentiation was inhibited by constitutively active Notch1-1C, encoding a truncated intracellular form of Notch 1 (FIG. 1A, FIG. 1B). Neither FoxO1-ADA nor Notch1-1 affected C_2C_{12} proliferation (FIG. 1I).

[0142] To determine whether the effect of FoxO1-ADA could be blocked by inhibition of endogenous signaling, a truncated Notch1 receptor lacking the transmembrane anchor and intracellular domain, which acts as a decoy receptor by binding Notch ligands (Nickoloff et al., 2003, Oncogene 22:6598-6608; Nickoloff et al., 2002, Cell Death Differ. 9:842-855). The Notch decoy partly rescued FoxO1-ADA inhibition of myoblast differentiation (FIG. 1A) and restored myosin expression to about 30% of control values (FIG. 1B). The decoy did not affect the ability of the C_2C_{12} cells to undergo differentiation and activate myosin expression in response to growth factor withdrawal (FIG. 1A, FIG. 1B). As an alternative to block Notch signaling, a presenilin inhibitor (PSE), compound E (Pan et al., 2004, Dev. Cell 7:731-743) was used. This reagent also rescued FoxO1-ADA inhibition of myoblast differentiation (FIG. 1A). To examine the role of FoxO1 on Notch signaling, FoxO1 siRNA and Notch-1C were co-transfected into cells. FoxO1 siRNA rescued inhibition of myoblast differentiation and myosin expression by Notch 1-Ic (FIG. 1A, FIG. 1B), while control siRNA had no effect. To rule out non-specific effects of FoxO1 siRNA on myoblast differentiation, a siRNA-resistant FoxO1-ADA was generated (FIG. 1J). FoxO1 siRNA failed to rescue inhibition of C2C12 differentiation caused by siRNA-resistant FoxO1-ADA (FIG. 1A). A quantitative analysis of these data is demonstrated in FIG. 1B, showing that Foxo1 and Notch1-IC decreased myosin levels by >80%, while Notch decoy and Foxo1 siRNA restored them to ~70% of fully differentiated cells. Similar data was obtained by performing a morphometric analysis of Myosin-positive

cells (FIG. 1K). These data indicate that Foxo1 is required for the effect of Notch on myoblast differentiation.

[0143] To test whether FoxO1 affected differentiation via its transcriptional function, a DNA binding deficient mutant (DBD-FoxO1ADA) was generated in the backbone of the ADA mutant bu replacement of N208A and H212R (Liu et al., 2005; Dowell et al., 2003, J. Biol. Chem. 278:45485-45491). This mutant is unable to bind DNA by measuring *Igfbp1* promoter activity, a canonical Foxo1 target. Foxo1-ADA increased *Igfbp1* promoter activity by 10-fold, whereas DBD-Foxo1ADA was unable to do so (FIG. 1L, left panel). Surprisingly, this mutant was as effective as the DNA –binding competent FoxO1-ADA at inhibiting differentiation (FIG. 1A). These data indicate that FoxO1 controls differentiation independently of its ability to bind DNA in a sequence specific manner.

Example 3: Altered Pattern of Muscle Differentiation Markers in FoxO$^+$ Embryos

[0144] To test the *in vivo* relevance of observations in cultured cells, the expression patterns of muscle differentiation markers were studied in FoxO1$^{-/-}$ embryos. FoxO1$^{-/-}$ embryos die at embryonic day 10.5 before muscle differentiation is complete. The findings disclosed *supra* indicate that FoxO1 inhibits muscle differentiation through suppression of MyoD. Therefore, it was expected that differentiation would be accelerated in the absence of FoxO1. Consistent with this expectation, *myosin* (*Mhc*) expression began at E10.5 in wild-type embryos, while *MyoD* mRNA began to rise in levels of expression at E8.5 and *myf5* was expressed at constant levels during this embryonic phase (FIG. 1E). Notch reportedly controls myoblast differentiation by inhibiting MyoD, without affecting Myf5. *MyoD* and *Myf5* expression were assayed in *FoxO1*$^{-/-}$ embryos and controls using real time RT-PCR. *MyoD* expression increased by about threefold in *FoxO1*$^{-/-}$ embryos and E8.5 and E9.5, whereas *Myf5* was unaffected (FIG. 1F). These results indicate that FoxO1 inhibits muscle differentiation through suppression of *MyoD in vivo*. Notch-IC has been reported to promote expression of Notch ligands and receptors (Shawber et al, 2003, Ann. N. Y. Acad. Sci. 995:162-70). Consistent with this finding, it was observed that Notch1-IC increased expression of the Notch ligand Jagged 1, and of the Notch1 and Notch2 receptors. FoxO1-ADA induced a similar increase (FIG. 1D). To determine whether the effect of FoxO1-ADA can be blocked by inhibition of endogenous Notch signaling, a truncated Notch1

receptor lacking the transmembrane anchor and intracellular domain was used to act as a decoy receptor by binding Notch ligands (U.S. Patent Application 20060030694). This mutant (termed "Notch decoy") partially rescued FoxO1-ADA as well as Notch1-IC inhibition of myoblast differentiation (FIG. 1A) and restored myosin expression to about 30% of control values (FIG. 1B). The partial ability of the Notch decoy to rescue differentiation is consistent with Notch acting on a ligand synthesized in response to Notch1-IC and FoxO1-ADA.

[0145] To examine the role of FoxO1 on Notch inhibition of myoblast differentiation, FoxO1 expression was inhibited in C_2C_{12} cells using small interfering RNA (siRNA). Fox O1 siRNA effectively suppressed expression of both transfected FLAG-FoxO1 (FIG. 1E) and endogenous FoxO1 (FIG. 1F) in a dose-dependent manner without affecting control proteins or other FoxO isoforms (FIG. 1G). Fox O1 siRNA partially rescued inhibition of myoblast differentiation by Notch1-IC (FIG. 1A), while the control SiRNA had no effect on myoblast inhibition. These data indicate that FoxO1 is required for the inhibitory effect of Notch on myoblast differentiation. According, compounds that inhibit FoxO1 expression or activity are useful for promoting myoblast differentiation.

Example 4: FoxO1 Binds to Csl and is Recruited to the *Hes1* Promoter

[0146] Notch1-IC binds to and co-activates Csl to promote *Hes1* and *Hey1* expression (Lai, 2002, EMBO Rep 3:840-5). To examine whether FoxO1 interacts with Csl in a Notch-dependent manner, C_2C_{12} cells expressing Notch1 receptor were co-cultured with HEK293 cells expressing the Notch ligand Jagged1 (denoted by the "+" sign), or LacZ as a negative control (denoted by the "-" sign). Several methods were used to investigate whether FoxO1 and Csl interact in the cultured cells.

[0147] Endogenous FoxO1 was detected in endogenous Csl immunoprecipitates (FIG. 2A). The co-immunoprecipitation was significantly enhanced by activation of Notch signaling.

[0148] To confirm the specificity of the interaction between FoxO1 and Csl, HA-tagged FoxO1 and FLAG-tagged Csl were co-expressed in C_2C_{12} cells. Following immunoprecipitation with anti-HA antiserum (to capture FoxO1), FLAG-Csl was detected

in the immunoblots (FIG. 2B). Conversely, following immunoprecipitation with anti-FLAG antiserum (to capture Csl), HA-FoxO1 was detected in the immunoblots (FIG. 2C). These data demonstrate an interaction between FoxO1 and Csl.

[0149] The efficiency of the Csl/FoxO1 co-immunoprecipitation with that of Csl/Notch1-IC was examined. In these experiments, Notch1-IC (FIG. 2D) and FoxO1 (FIG. 2E) were detected in similar amounts in Csl immunoprecipitates, consistent with the interpretation that FoxO1 and Notch bind to Csl with similar affinities. The ability to co-immunoprecipitate with Csl appears to be specific to FoxO1, as other FoxO isoforms were not detected in Csl immunoprecipitates (FIG. 2L). A truncated FoxO1 mutant (Δ256, encoding amino acids 1-256; Nakae et al., 2001, J. Clin. Invest. 108:1359-1367) retained the ability to interact with Csl. FLAG-Csl was detected in Myc-Δ256 immunoprecipitates (FIG. 2F), and HA-Δ256 was detected in FLAG-Csl immunoprecipitates (FIG. 2G), indicating that Csl interacts with FoxO1's N-terminal domain.

[0150] To determine whether there is a direct protein interaction between FoxO1 and Csl and to map the interaction domain(s), pull down assays were carried out using affinity-purified GST-FoxO1 that was produced in bacteria and FLAG-Csl expressed in HEK293 cells. Csl associated with full-length and N-terminal FoxO1 (amino acids 1-300), but not with C-terminal FoxO1 (amino acids 290-655) or GST (FIG. 2H).

[0151] Next, the Csl domain that interacts with FoxO1 was mapped using a cell-free system with GST-FoxO1 and GST-FLAG/Csl purified from bacterial cultures. Full-length (1-655) and N-terminal (1-300) FoxO1 was recovered from Csl immunoprecipitates, however C-terminal (290-655) FoxO1 was not recovered. Conversely, N-terminal FoxO1 was found to interact with full-length and N-terminal Csl (FIG. 2I). Csl deletion mutants were used to map the Foxo1-binding domain in Csl. More detailed mapping of this region indicated that FoxO1 binds to a domain encompassing amino acids 172-279 of Csl (FIG. 2J). This region contains the Csl corepressor-binding domain (Hsieh et al., Science, 1995, 268:560-563; Kao et al., 1998, Genes Dev. 12:2269-2277) (FIG. 2J, diagram). Interestingly, this domain is required for DNA and corepressor binding, but does not contribute to Notch binding (38, 39).

[0152] Csl binds to a consensus sequence in the *Hes1* promoter (Tun et al., 1994, Nucleic Acids Res. 22:965-971), which thus provides a useful readout assay of the

FoxO1/Csl interaction. If the latter were required to regulate C_2C_{12} differentiation, three predictions should be met: (a) FoxO1 should be detected in chromatin immunoprecipitation assays (ChIP) spanning the Csl element in the *Hes1* promoter, (b) the interaction should be differentiation-dependent and (c) inhibition of differentiation by FoxO1-ADA should be accompanied by constitutive binding to the Csl element in the *Hes1* promoter. FIG. 2K demonstrates that all predictions are fulfilled. ChIPs were performed using primers spanning the Csl binding site of *Hes1* in differentiating C_2C_{12} cells. Endogenous FoxO1, Notch1, and Csl were detected in immunoprecipitates from undifferentiated cells (FIG. 2K, Endog. lanes, Day 0). As the PCR-amplified sequence contains no forkhead binding sites, it was concluded that FoxO1 binds to this DNA fragment via Csl. Moreover, binding of both FoxO1 and Notch 1 decreased as cells became differentiated (day 1 and day 2). When cells were transduced with constitutively nuclear FoxO1-ADA, differentiation was inhibited FIG. 1A and mutant FoxO1 was persistently bound to the *Hes1* promoter, as were both Csl and Notch1 (FIG. 2K, FoxO1-ADA lanes).

[0153] Correlated with the decrease in FoxO1 and Notch 1 binding to *Hes1* promoter, Hes1 mRNA expression decreased, while myosin protein levels increased during myoblast differentiation (FIG. 2K). To rule out a direct effect of FoxO1 on Csl transcription, reporter gene assays were carried out with the *Csl* promoter. FoxO1 failed to activate expression of a Csl reporter gene, despite the presence of ten repeats of a forkhead binding site in the Csl promoter (Kawaichi et al., 1992, J. Biol. Chem. 267:4016-4022) (FIG. 2M). Moreover, Csl expression was unaffected in C_2C_{12} cells expressing FoxO1-ADA. These data indicated that FoxO1 regulates Notch-dependent differentiation via protein/protein interactions with Csl.

Example 5: FoxO1 is Required for Notch Induction of *Hes1* and *Hey* 1 genes Via Csl

[0154] The ability of Foxo1-ADA to promote expression of endogenous *Hes1*, *Hes5* and *Hey1* was examined in C2C12 cells. Both Foxo1-ADA and Notch1-IC increased the expression of the three genes, while Foxo1 siRNA inhibited *Hes1*, *Hes5* and *Hey1* expression induced by Notch1-IC (FIG. 3B). Foxo1 siRNA had no effect on *Hes1*, *Hes5* and *Hey1* expression in growth factor-deprived cells (FIG. 3B).

[0155] *Hes1* can be considered a prototypical Notch target gene. The ability of FoxO1 to regulate *Hes1* transcription was investigated using reporter assays with the *Hes1* promoter, as well as by measuring of *Hes1* expression. It was found that FoxO1-ADA and Notch1-IC induced Hes1 promoter activity by 1.8- and 2.5- fold, respectively. Co-transfection of FoxO1-ADA with Notch1-IC caused a 2.5-fold increase in Hes1 promoter activity (FIG. 3A). Co-transfection of FoxO1 siRNA with Notch1-IC suppressed Notch-induced *Hes1* activity in a dose-dependent manner, while control siRNA had no effect (FIG. 3A). Similar results were obtained with a synthetic *Hes1* reporter containing four tandem repeats of the Csl binding motif (FIG. 3C). Moreover, DBD-FoxO1 ADA induced *Hes1* reporter gene activity to an even greater extent than FoxO1 -ADA, confirming that direct DNA binding is not required for FoxO1 activation of Hes1 (FIG. 3D).

[0156] The ability of FoxO1-ADA to promote expression of endogenous *Hes1* in C_2C_{12} cells was also examined. In these experiments, both FoxO1-ADA and Notch1-IC increased *Hes1* expression, while FoxO1 siRNA inhibited *Hes1* expression induced by Notch1-IC (FIG. 3B). FoxO1 siRNA had no effect on either Hes1 activity or expression in growth factor-deprived cells (FIG. 3A and FIG. 3B).

[0157] The failure of Notch1-IC to induce *Hes1* expression in cells expressing FoxO1 siRNA suggests that FoxO1 is required for the Csl/Notch interaction. Thus, the binding of FoxO1 and Notch1 to the *Hes1* promoter was examined. These experiments were performed using a co-culture system in which C_2C_{12} cells expressing Notch1 were co-cultured with HEK293 cells expressing Jagged1 to induce activation of endogenous Notch signaling. Co-culture in the presence of Jagged1-expressing cells increased endogenous FoxO1 (FIG. 4A, lanes 1-2) and Notch1 binding to the *Hes1* promoter in ChIP assays (FIG. 4A and FIG. 4B, lanes 1-2) (Fryer et al., 2004, Mol. Cell 16:509-520 (2004). These data are consistent with the observation that FoxO1 co-immunoprecipitation with Csl increased upon co-culture (FIG. 2A). To test whether FoxO1 binding to the *Hes1* promoter is Csl-dependent, Csl expression was inhibited with siRNA (FIG. 4G). Transfection of Csl siRNA inhibited both FoxO1 and Notch1 binding to *Hes1* promoter (FIG. 4A, lanes 3-4), indicating that they are Csl-dependent. Moreover, FoxO1-ADA failed to induce *Hes1* expression in the presence of Csl siRNA (FIG. 3B, lane 5).

[0158] The results of the ChIP experiments were corroborated by *Hes1* promoter assays. In these experiments, it was found that expression of Jagged1 or Notch1 alone had no effect

on *Hes1* activity, but co-culturing yielded a 3.7-fold increase in *Hes1* reporter gene activity (FIG. 4C). FoxO1 siRNA abolished Notch binding to the *Hes1* promoter in ChIP assays (FIG. 4B, lanes 3-4) and induction of *Hes1* promoter activity (FIG. 4C). These results indicate that FoxO1 is required for binding of Notch1 to the *Hes1* promoter, and suggest a mechanism whereby inhibition of FoxO1 expression restores differentiation of myoblasts expressing Notch1-IC. The ability of FoxO1 siRNA to inhibit Notch induction of *Hes1* in a co-culture system indicates that the effects observed in differentiation experiments with Notch1-IC are not due to non-physiologic activation of Notch signaling by the truncated intracellular Notch1 mutant.

Example 6: Foxo1 promotes corepressor clearance and Maml1 binding to Csl

[0159] To further investigate the molecular mechanism of FoxO1-dependent activation of *Hes1* expression, corepressor/coactivator exchange at the *Hes1* promoter was assayed. Activation of Notch signaling released the corepressors NcoR and SMRT (Liang et al, 2002, Genes Dev. 16:1977-1989) and recruited the coactivator MAML1 (Fryer et al., 2004, *supra*) to the *Hes1* promoter. FoxO1 siRNA prevented Notch-induced corepressor exchange (FIG. 4D). These data are consistent with the observation that FoxO1 binds to the region of amino acids 172-279 of Csl (FIG. 2J), which has been shown to contain the NcoR/SMRT binding sites (Hsieh et al., 1995, Science 268:560-563; Kao et al., 1998, Genes Dev. 12:2269-2277).

[0160] To demonstrate that the observed changes in the transcriptional complex result in changes in *Hes1* activity, expression of Hes1 target genes involved in myogenesis was investigated. The expression of Hes1 target genes was investigated in myogenesis. Hes1 suppresses myoblast differentiation by inhibiting the bHLH transcription factor MyoD, without affecting Myf5 (16, 17). Expression analyses revealed that Notch1-IC or FoxO1-ADA suppressed *MyoD*, while *Myf5* was unaffected. Notch decoy or FoxO1 siRNA partially restored *MyoD* expression (FIG. 4E). These data are consistent with the observation that *MyoD* expression is increased in *FoxOT1*$^{-/-}$ embryos, whereas *Myf5* expression is normal (FIG. 4F).

Example 7: Interaction of FoxO1 and Notch in Neural, Adipose, and Endothelial Cell Differentiation

[0161] The question of whether the interaction of FoxO1 and Notch signaling is unique to myoblast differentiation or if the interaction occurs in other Notch-dependent cell types. Because Notch affects neural development, PC12 cell differentiation was investigated. In this cell type, exposure to nerve growth factor (NGF) causes neurite sprouting and differentiation into axons and dendrites. In NGF-treated PC12 cells, differentiative effects were observed in about55% of the cells. Transduction of PC12 cells with either FoxO1-ADA or Notch1-IC reduced the number of neurite-bearing cells after NGF induction to 4% and 7%, respectively. Notch decoy restored neurite outgrowth to 50% of control levels in cells transduced with FoxO1-ADA and induced with NGF. FoxO1 siRNA restored neurite outgrowth to 53% of control in cells transduced with Notch1-IC (FIG. 5A and FIG. 5D).

[0162] Similar experiments were performed to examine the interaction of FoxO1 and Notch signaling in adipocyte differentiation using 3T3-F442A pre-adipocytes. Transduction of FoxO1-ADA or Notch1-IC inhibits adipocyte differentiation (Ross et al., 2004, Mol. Cell. Biol. 24:3505-3513; Nakae et al., 2003, Dev. Cell 4:19-29). Both Notch decoy and FoxO1 siRNA partially restored differentiation inhibited by FoxO1-ADA or Notch1-IC (FIG. 5B and FIG. 5E).

[0163] FoxO/Notch interaction was also studied in an angiogenesis model. In these experiments, collagen sandwich assays were performed with human umbilical vein endothelial cells (HUVEC). Formation of a capillary-like network was inhibited to a similar extent by either FoxO1-ADA or Notch1-IC. Notch inhibition by PSI provided partial rescue of FoxO1-ADA inhibition (FIG. 5C and FIG. 5F). From these data, it can be concluded that the FoxO/Notch interaction is a general mechanism to control cell differentiation.

Example 8: Altered fiber type composition in skeletal muscle lacking Foxo1

[0164] Based on the cellular data, Foxo1 function was probed in muscle differentiation *in vivo* using conditional gene inactivation. The predicted outcome of this experiment is accelerated differentiation of MyoD-containing, but not Myf5-containing myoblasts. Because MyoD is the predominant myogenic factor in fast fibers, while Myogenin is

predominant factor in slow fibers (44), the removal of Foxo/Notch inhibition on MyoD expression should result in increased formation of fast fibers, potentially at the expense of slow fibers.

[0165] There are three Foxo isoforms in mice: Foxo1, 3, and 4 (8, 9). The latter is predominant in most muscle types (45) except soleus, where Foxo1 is the most abundant (FIG. 6A). Coincidentally, soleus is also physiologically enriched in slow-twitch fibers, and thus readily allowed to test the hypothesis. Foxo1 expression was inactivated in skeletal muscle by crossing mice homozygous for a floxed *Foxo1* allele with Myogenin-cre transgenics. mRNA analysis indicated that the knockout occurred as planned (data not shown). Histological analyses revealed a reduction of type I (slow-twitch) fibers in soleus of Myog-Foxo1 mice, while type II fiber-enriched muscles were unaffected (FIG. 6B). Consistent with the histological findings, expression of type I fiber markers decreased, while type II fiber markers increased in Myog-Foxo1 mice (FIG. 6C). Expression of the myogenic transcription factors MyoD, Myf5 and Myogenin was then analyzed. MyoD is the predominant factor in fast fibers, and Myogenin in slow fibers (44). Consistent with the histopathology, a twofold increase was found in MyoD expression and an ~80% decrease in Myogenin, while Myf5 expression was unchanged (FIG. 6C). Moreover, expression the Foxo1 coactivator Pgc1α, which regulates type I fiber determination (30) was unchanged, indicating that the phenotype of Myog-Foxo1 mice cannot be accounted for by decreased Foxo1-dependent Pgc1α transcription (FIG. 6C) (46). As a functional correlate of the observed fiber type switch, running performance was examined on a treadmill. Indeed, Myog-Foxo1 mice displayed reduced running capacity, as predicted from the reduction in type I (endurance) fibers (FIG. 6D).

[0166] Finally, to determine whether these changes reflected developmental alterations in fiber-type specification, as opposed to adaptive or cell-nonautonomous factors, MyoD expression was determined in Foxo1 (24) and Notch1 knockout (25) embryos at E9.5. In $Foxo1^{-/-}$ embryos, MyoD levels increased 3.1±1.1 fold, and in $Notch1^{-/-}$ embryos 7.3±2.9 fold compared to controls ($P<0.05$ in both mutants vs. wildtype, n=4). The increase in MyoD expression observed *in vivo* is consistent with the physical and functional interactions between Foxo1 and Notch at this key signaling nexus in myoblast differentiation. Thus, the fiber-type switch in Myog-Foxo1 mice is purportedly the result of accelerated differentiation of MyoD-containing myoblasts during embryonic development.

[0167] The EXAMPLES above provide biochemical, cellular and genetic evidence that Foxo and Notch pathways cooperate in the regulation of muscle differentiation. The data reveal a novel mode of Foxo1 action to promote corepressor exchange at the *Hes1* promoter via direct binding to the Csl NTD region (FIG. 4F). We propose that Foxo1 binding to this domain stabilizes the Notch/Csl complex and promotes corepressor clearance and Maml1 recruitment, consistent with the proposed role of NTD from structural studies (37). The findings also provide a mechanism by which two major biochemical pathways, the phosphoinositol-3-kinase/Akt pathway and the Notch/Hes pathway, converge in a synergistic manner to control cellular differentiation *in vivo*.

[0168] The proposed role for Foxo1 is independent of its transcriptional function, and involves a direct interaction with Csl. While these studies have focused on Hes-1 as a prototypical effector of Notch1 signaling, this data should not be construed to indicate that Hes-1 is the sole mediator of the Notch/Foxo interaction. For example, a similar Foxo/Notch epistasis was observed in the differentiation of pre-adipocytes, PC-12, and HUVECs, suggesting that Foxo interacts with Notch in multiple cell contexts (data not shown). It is proposed that Notch/Foxo cooperation integrates environmental cues through Notch with metabolic cues through Foxo1 to regulate progenitor cell maintenance and differentiation. This two-tiered mechanism allows committed progenitor cells in various tissues to avoid differentiation in response to developmental cues (Notch) when Foxo1 is active, i.e., in the absence of growth factors. These cells would then persist in a dormant state in adult tissues, where they can terminally differentiate in response to a combination of Notch ligand and hormonal/nutritional cues leading to Foxo1 inhibition. This interpretation is consistent with the fiber-type switch observed in Foxo1-deficient muscle, an observation that appears to position Foxo1 as a fate decider within the myogenic lineage, as opposed to an inducer of the myogenic program. It remains to be seen whether other Foxo and Notch isoforms also interact and how they contribute to this process.

[0169] The demonstration that Foxo1 is a coregulator of gene expression provides a potential explanation for the protean functions of this transcription factor. An interesting question emerging from our studies is how the switch from one function to the other is effected, and how the complex post-translational modifications of Foxo1 in response to growth factors, hormones and nutrients impinge on this process. The findings have broad

implications for the pathophysiology of disease processes that involve Foxo1 signaling. A potential implication of this observation is the ability to explore the use of agents that inhibit Notch signaling (47) as a treatment of metabolic disorders characterized by excessive Foxo function (48).

References

1. Singec, I., Jandial, R., Crain, A., Nikkhah, G., and Snyder, E.Y. 2007. The leading edge of stem cell therapeutics. *Annu Rev Med* 58:313-328.

2. Accili, D., and Arden, K.C. 2004. FoxOs at the Crossroads of Cellular Metabolism, Differentiation, and Transformation. *Cell* 117:421-426.

3. Hribal, M.L., Nakae, J., Kitamura, T., Shutter, J.R., and Accili, D. 2003. Regulation of insulin-like growth factor-dependent myoblast differentiation by Foxo forkhead transcription factors. *J Cell Biol* 162:535-541.

4. Nakae, J., Kitamura, T., Kitamura, Y., Biggs, W.H., Arden, K.C., and Accili, D. 2003. The forkhead transcription factor foxo1 regulates adipocyte differentiation. *Dev Cell* 4:119-129.

5. Potente, M., Urbich, C., Sasaki, K.I., Hofmann, W.K., Heeschen, C., Aicher, A., Kollipara, R., Depinho, R.A., Zeiher, A.M., and Dimmeler, S. 2005. Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. *J Clin Invest* 115:2382-2392.

6. Liu, Z.P., Wang, Z., Yanagisawa, H., and Olson, E.N. 2005. Phenotypic modulation of smooth muscle cells through interaction of Foxo4 and myocardin. *Dev Cell* 9:261-270.

7. Castrillon, D.H., Miao, L., Kollipara, R., Horner, J.W., and DePinho, R.A. 2003. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science* 301:215-218.

8. Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C., et al. 2007. FoxOs

Are Critical Mediators of Hematopoietic Stem Cell Resistance to Physiologic Oxidative Stress. *Cell* 128:325-339.

9. Paik, J.H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J.W., Carrasco, D.R., et al. 2007. FoxOs Are Lineage-Restricted Redundant Tumor Suppressors and Regulate Endothelial Cell Homeostasis. *Cell* 128:309-323.

10. Shawber, C.J., and Kitajewski, J. 2004. Notch function in the vasculature: insights from zebrafish, mouse and man. *Bioessays* 26:225-234.

11. Lai, E.C. 2002. Keeping a good pathway down: transcriptional repression of Notch pathway target genes by CSL proteins. *EMBO Rep* 3:840-845.

12. Jensen, J., Pedersen, E.E., Galante, P., Hald, J., Heller, R.S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P., and Madsen, O.D. 2000. Control of endodermal endocrine development by Hes-1. *Nat Genet* 24:36-44.

13. Ross, D.A., Rao, P.K., and Kadesch, T. 2004. Dual roles for the Notch target gene Hes-1 in the differentiation of 3T3-L1 preadipocytes. *Mol Cell Biol* 24:3505-3513.

14. Ohtsuka, T., Ishibashi, M., Gradwohl, G., Nakanishi, S., Guillemot, F., and Kageyama, R. 1999. Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. *Embo J* 18:2196-2207.

15. Shawber, C., Nofziger, D., Hsieh, J.J., Lindsell, C., Bogler, O., Hayward, D., and Weinmaster, G. 1996. Notch signaling inhibits muscle cell differentiation through a CBF1-independent pathway. *Development* 122:3765-3773.

16. Sasai, Y., Kageyama, R., Tagawa, Y., Shigemoto, R., and Nakanishi, S. 1992. Two mammalian helix-loop-helix factors structurally related to Drosophila hairy and Enhancer of split. *Genes Dev* 6:2620-2634.

17. Kuroda, K., Tani, S., Tamura, K., Minoguchi, S., Kurooka, H., and Honjo, T. 1999. Delta-induced Notch signaling mediated by RBP-J inhibits MyoD expression and myogenesis. *J Biol Chem* 274:7238-7244.

18. Nofziger, D., Miyamoto, A., Lyons, K.M., and Weinmaster, G. 1999. Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts. *Development* 126:1689-1702.

19. Wilson-Rawls, J., Molkentin, J.D., Black, B.L., and Olson, E.N. 1999. Activated notch inhibits myogenic activity of the MADS-Box transcription factor myocyte enhancer factor 2C. *Mol Cell Biol* 19:2853-2862.

20. Hirsinger, E., Malapert, P., Dubrulle, J., Delfini, M.C., Duprez, D., Henrique, D., Ish-Horowicz, D., and Pourquie, O. 2001. Notch signalling acts in postmitotic avian myogenic cells to control MyoD activation. *Development* 128:107-116.

21. Conboy, I.M., and Rando, T.A. 2002. The regulation of Notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Dev Cell* 3:397-409.

22. Shawber, C.J., Das, I., Francisco, E., and Kitajewski, J. 2003. Notch signaling in primary endothelial cells. *Ann N Y Acad Sci* 995:162-170.

23. Limbourg, F.P., Takeshita, K., Radtke, F., Bronson, R.T., Chin, M.T., and Liao, J.K. 2005. Essential role of endothelial Notch1 in angiogenesis. *Circulation* 111:1826-1832.

24. Hosaka, T., Biggs, W.H., 3rd, Tieu, D., Boyer, A.D., Varki, N.M., Cavenee, W.K., and Arden, K.C. 2004. Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. *Proc Natl Acad Sci U S A* 101:2975-2980.

25. Krebs, L.T., Xue, Y., Norton, C.R., Shutter, J.R., Maguire, M., Sundberg, J.P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R., et al. 2000. Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev* 14:1343-1352.

26. McKinsey, T.A., Zhang, C.L., and Olson, E.N. 2001. Control of muscle development by dueling HATs and HDACs. *Curr Opin Genet Dev* 11:497-504.

27. Rudnicki, M.A., and Jaenisch, R. 1995. The MyoD family of transcription factors and skeletal myogenesis. *Bioessays* 17:203-209.

28. Bassel-Duby, R., and Olson, E.N. 2006. Signaling pathways in skeletal muscle remodeling. *Annu Rev Biochem* 75:19-37.

29. Schiaffino, S., and Reggiani, C. 1996. Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol Rev* 76:371-423.

30. Lin, J., Wu, H., Tarr, P.T., Zhang, C.Y., Wu, Z., Boss, O., Michael, L.F., Puigserver, P., Isotani, E., Olson, E.N., et al. 2002. Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature* 418:797-801.

31. Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P., et al. 2006. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* 127:1109-1122.

32. Nickoloff, B.J., Osborne, B.A., and Miele, L. 2003. Notch signaling as a therapeutic target in cancer: a new approach to the development of cell fate modifying agents. *Oncogene* 22:6598-6608.

33. Nickoloff, B.J., Qin, J.Z., Chaturvedi, V., Denning, M.F., Bonish, B., and Miele, L. 2002. Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPARgamma. *Cell Death Differ* 9:842-855.

34. Pan, Y., Lin, M.H., Tian, X., Cheng, H.T., Gridley, T., Shen, J., and Kopan, R. 2004. gamma-secretase functions through Notch signaling to maintain skin appendages but is not required for their patterning or initial morphogenesis. *Dev Cell* 7:731-743.

35. Dowell, P., Otto, T.C., Adi, S., and Lane, M.D. 2003. Convergence of peroxisome proliferator-activated receptor gamma and Foxo1 signaling pathways. *J Biol Chem* 278:45485-45491.

36. Nakae, J., Kitamura, T., Silver, D.L., and Accili, D. 2001. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J Clin Invest* 108:1359-1367.

37. Kovall, R.A., and Hendrickson, W.A. 2004. Crystal structure of the nuclear effector of Notch signaling, CSL, bound to DNA. *Embo J* 23:3441-3451.

38. Hsieh, J.J., and Hayward, S.D. 1995. Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2. *Science* 268:560-563.

39. Kao, H.Y., Ordentlich, P., Koyano-Nakagawa, N., Tang, Z., Downes, M., Kintner, C.R., Evans, R.M., and Kadesch, T. 1998. A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. *Genes Dev* 12:2269-2277.

40. Tun, T., Hamaguchi, Y., Matsunami, N., Furukawa, T., Honjo, T., and Kawaichi, M. 1994. Recognition sequence of a highly conserved DNA binding protein RBP-J kappa. *Nucleic Acids Res* 22:965-971.

41. Kawaichi, M., Oka, C., Shibayama, S., Koromilas, A.E., Matsunami, N., Hamaguchi, Y., and Honjo, T. 1992. Genomic organization of mouse J kappa recombination signal binding protein (RBP-J kappa) gene. *J Biol Chem* 267:4016-4022.

42. Fryer, C.J., White, J.B., and Jones, K.A. 2004. Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Mol Cell* 16:509-520.

43. Liang, Y., Chang, J., Lynch, S.J., Lukac, D.M., and Ganem, D. 2002. The lytic switch protein of KSHV activates gene expression via functional interaction with RBP-Jkappa (CSL), the target of the Notch signaling pathway. *Genes Dev* 16:1977-1989.

44. Hughes, S.M., Taylor, J.M., Tapscott, S.J., Gurley, C.M., Carter, W.J., and Peterson, C.A. 1993. Selective accumulation of MyoD and myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. *Development* 118:1137-1147.

45. Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., Biggs, W.H., 3rd, Wright, C.V., White, M.F., Arden, K.C., and Accili, D. 2002. The forkhead transcription factor

Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. *J Clin Invest* 110:1839-1847.

46. Daitoku, H., Yamagata, K., Matsuzaki, H., Hatta, M., and Fukamizu, A. 2003. Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. *Diabetes* 52:642-649.

47. Miele, L., Miao, H., and Nickoloff, B.J. 2006. NOTCH signaling as a novel cancer therapeutic target. *Curr Cancer Drug Targets* 6:313-323.

48. Accili, D. 2004. Lilly lecture 2003: the struggle for mastery in insulin action: from triumvirate to republic. *Diabetes* 53:1633-1642.

49. Knapp, J.R., Davie, J.K., Myer, A., Meadows, E., Olson, E.N., and Klein, W.H. 2006. Loss of myogenin in postnatal life leads to normal skeletal muscle but reduced body size. *Development* 133:601-610.

50. Ogilvie, R.W., and Feeback, D.L. 1990. A metachromatic dye-ATPase method for the simultaneous identification of skeletal muscle fiber types I, IIA, IIB and IIC. *Stain Technol* 65:231-241.

51. Das, I., Craig, C., Funahashi, Y., Jung, K.M., Kim, T.W., Byers, R., Weng, A.P., Kutok, J.L., Aster, J.C., and Kitajewski, J. 2004. Notch oncoproteins depend on gamma-secretase/presenilin activity for processing and function. *J Biol Chem* 279:30771-30780.

52. Chi, T., Yan, Z., Xue, Y., and Wang, W. 2004. Purification and functional analysis of the mammalian SWI/SNF-family of chromatin-remodeling complexes. *Methods Enzymol* 377:299-316.

53. Puigserver, P., Rhee, J., Donovan, J., Walkey, C.J., Yoon, J.C., Oriente, F., Kitamura, Y., Altomonte, J., Dong, H., Accili, D., et al. 2003. Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature* 423:550-555.

Other Embodiments

[0170] It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for identifying a compound capable of modulating Notch activity, the method comprising:

(a) contacting a FoxO1 polypeptide or fragment thereof with a Csl polypeptide or fragment thereof under protein binding conditions; and

(b) optionally, admixing a test compound with the polypeptides of (a); and

(c) determining whether the test compound inhibits the binding of FoxO1 polypeptide with Csl polypeptide when compared to the binding in the absence of the compound, wherein a test compound that can inhibit the binding is a compound capable of modulating Notch activity.

2. The method of claim 1, wherein the test compound is admixed before contacting, during contacting, after contacting, or any combination thereof.

3. The method of claim 1, wherein a fragment of a FoxO1 polypeptide binds a Csl polypeptide or a fragment thereof

4. The method of claim 3, wherein the Csl polypeptide or fragment thereof is at least about 500 base pairs in length.

5. The method of claim 3, wherein the Csl polypeptide or fragments comprises amino acids 179-272 of a full-length Csl protein.

6. The method of claim 1, wherein the FoxO1 polypeptide comprises from about 1 to about 300 contiguous amino acids from the amino terminus of a FoxO1 sequence.

7. The method of claim 1, wherein the FoxO1 polypeptide comprises from about 20 to about 300 contiguous amino acids from the amino terminus of a FoxO1 sequence.

8. The method of claim 1, further comprising determining whether the test compound modulates Hes1 expression in a cell.

9. The method of claim 1, further comprising determining whether the compound modulates expression of at least one of: an angiogenesis gene, a myogenesis gene, a

neurogenesis gene, a gut development gene, an adipogenic gene, or a pancreatic β cell differentiation gene

10. The method of claim 1, wherein the contacting occurs in a cell.

11. The method of claim 10, wherein the cell is a myoblast, a pre-adipocyte, a neuronal precursor cell, a PC-12 cell, a gut endoderm cell, an embryonic stem cell, or a pancreatic duct cell.

12. A method for identifying a compound capable of inhibiting binding of a FoxO1 protein and a Csl protein, the method comprising:

(a) admixing a compound with a FoxO1 polypeptide or a fragment thereof and a Csl polypeptide under protein-binding conditions; and

(b) measuring the amount of FoxO1 polypeptide bound to Csl,

wherein a decrease in the amount of FoxO1 bound to Csl in the presence of the compound compared to the amount of FoxO1 bound in the absence of the compound indicates that the compound is capable of inhibiting the interaction between Csl and FoxO1.

13. The method of claim 12, wherein the FoxO1 is a fragment of FoxO1 that can bind to Csl.

14. The method of claim 12, further comprising determining whether the test compound modulates Notch activity.

15. The method of claim 12, further comprising determining whether the test compound modulates *Hes1* expression in a cell.

16. The method of claim 12, further comprising determining whether the compound modulates expression of at least one of: an angiogenesis gene, a myogenesis gene, a neurogenesis gene, a gut development gene, an adipogenic gene, a pancreatic β cell differentiation gene, or any combination thereof

17. The method of claim 12, wherein the method further comprises determining whether the compound inhibits binding of the Csl protein to the N-terminal region of the FoxO1 protein.

18. The method of claim 12, wherein the method further comprises determining whether the compound binds to the N-terminal region of the FoxO1 protein.

19. The method of claim 12, wherein the method further comprises determining whether the test compound binds to the Csl protein.

20. The method of claim 19, wherein the method further comprises determining whether the test compound binds to all or a portion of amino acids 1-300 of a Csl protein.

21. The method of claim 19, wherein the method further comprises determining whether the test compound binds to all or a portion of amino acids 179-272 of a human Csl or a homolog thereof.

22. The method of any one of claims 13, 14, or 15, wherein the cell is a myoblast, a pre-adipocyte, a neuronal precursor cell, a PC-12 cell, a pancreatic β cell precursor, a gut endoderm cell, an embryonic stem cell, or a pancreatic duct cell.

23. A compound capable of inhibiting an interaction between FoxO1 and Csl comprising a peptide from about amino acid position 1 to about amino acid position 300 of a Fox01 protein, an antibody, an antibody fragment, a peptide, a peptoid, a non-nucleic acid small organic molecule, or other small molecule.

24. The compound of claim 23, wherein the peptide is from a protein sequences represented by Genbank accession no. NM_019739.

25. A method for inhibiting muscle differentiation, the method comprising contacting a myoblast with a compound that can enhance the interaction between FoxO1 and Csl.

26. The method of claim 25, wherein the myoblast is in a mammal.

27. The method of claim 226, wherein the mammal is a human.

28. The method of claim 25, wherein the compound comprises a FoxO1 polypeptide, a Csl-binding fragment of FoxO1 polypeptide, a nucleic acid encoding a FoxO1 polypeptide

or a fragment thereof, a Csl polypeptide, a FoxO1-binding fragment of a Csl polypeptide, a nucleic acid encoding a Csl polypeptide or fragment thereof, or any combination thereof.

29. A method for increasing muscle differentiation, the method comprising contacting a myoblast with a compound that can inhibit the interaction between FoxO1 and Csl.

30. The method of claim 29, wherein the myoblast is in a mammal.

31. The method of claim 230, wherein the mammal is a human.

32. A method of modulating differentiation, the method comprising contacting an undifferentiated cell with a compound that modulates the interaction between Csl and FoxO1.

33. The method of claim 32, wherein the compound inhibits the interaction between FoxO1 and Csl and differentiation is enhanced.

34. The method of claim 32, wherein the compound enhances myoblast differentiation, adipogenesis, angiogenesis, pancreatic β cell differentiation, or neurite sprouting.

35. The method of claim 32, wherein the compound enhances the interaction between FoxO1 and Csl and differentiation is decreased.

36. The method of claim 32, wherein the compound inhibits myoblast differentiation, angiogenesis, pancreatic β cell differentiation, or neurite sprouting.

37. A method of inhibiting expression of *Hes1*, the method comprising contacting a cell that can express *Hes1* with a compound that inhibits FoxO1 protein expression or activity.

38. The method of claim 37, wherein the compound comprises an siRNA or interfering RNA (RNAi) that specifically inhibits expression of a FoxO1 gene.

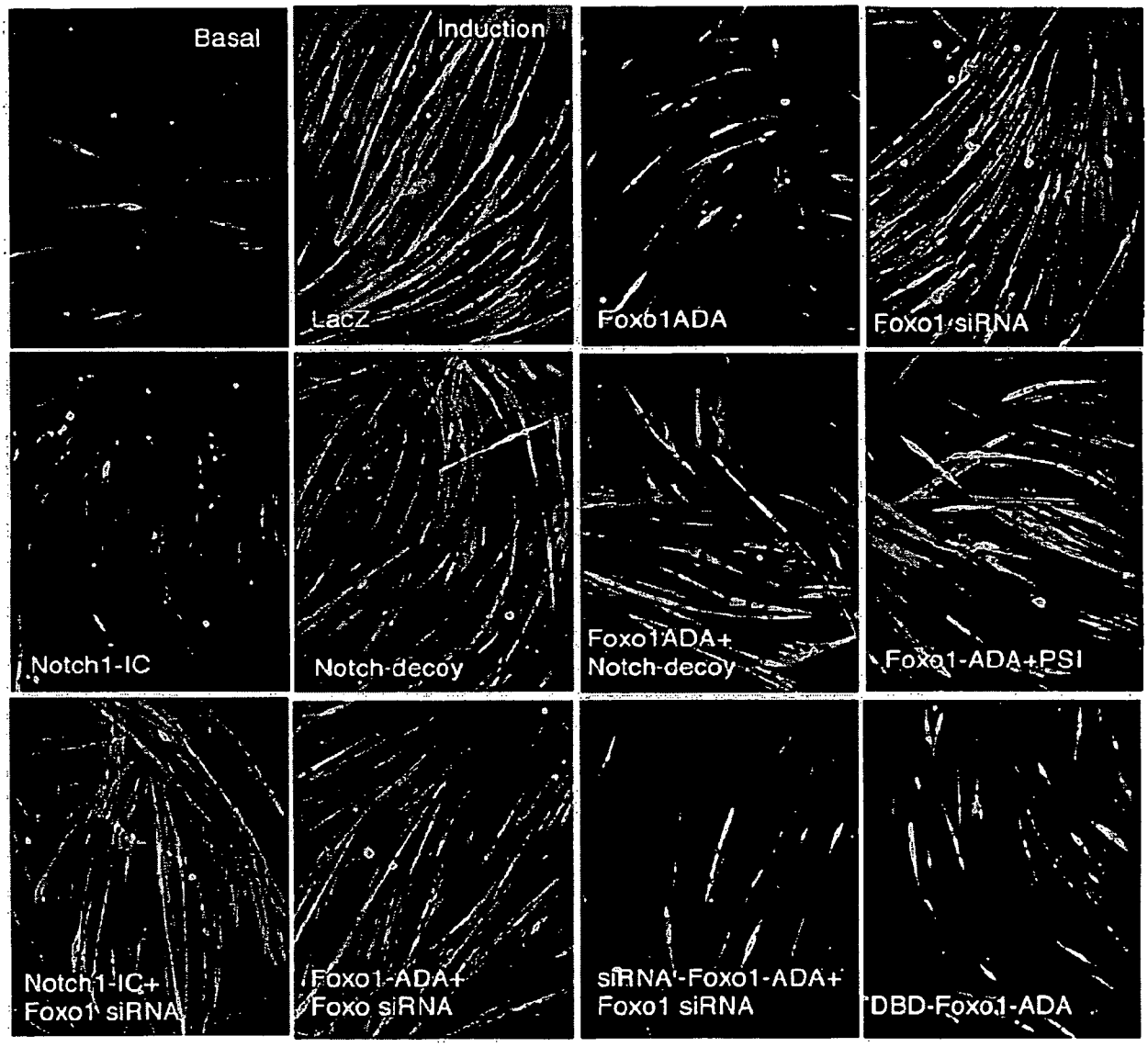

Basal
Induction
LacZ
Foxo1ADA
Foxo1 siRNA
Notch1-IC
Notch-decoy
Foxo1ADA+
Notch-decoy
Foxo1-ADA+PSI
Notch1-IC+
Foxo1 siRNA
Foxo1-ADA+
Foxo siRNA
siRNA-Foxo1-ADA+
Foxo1 siRNA
DBD-Foxo1-ADA

FIG. 1A

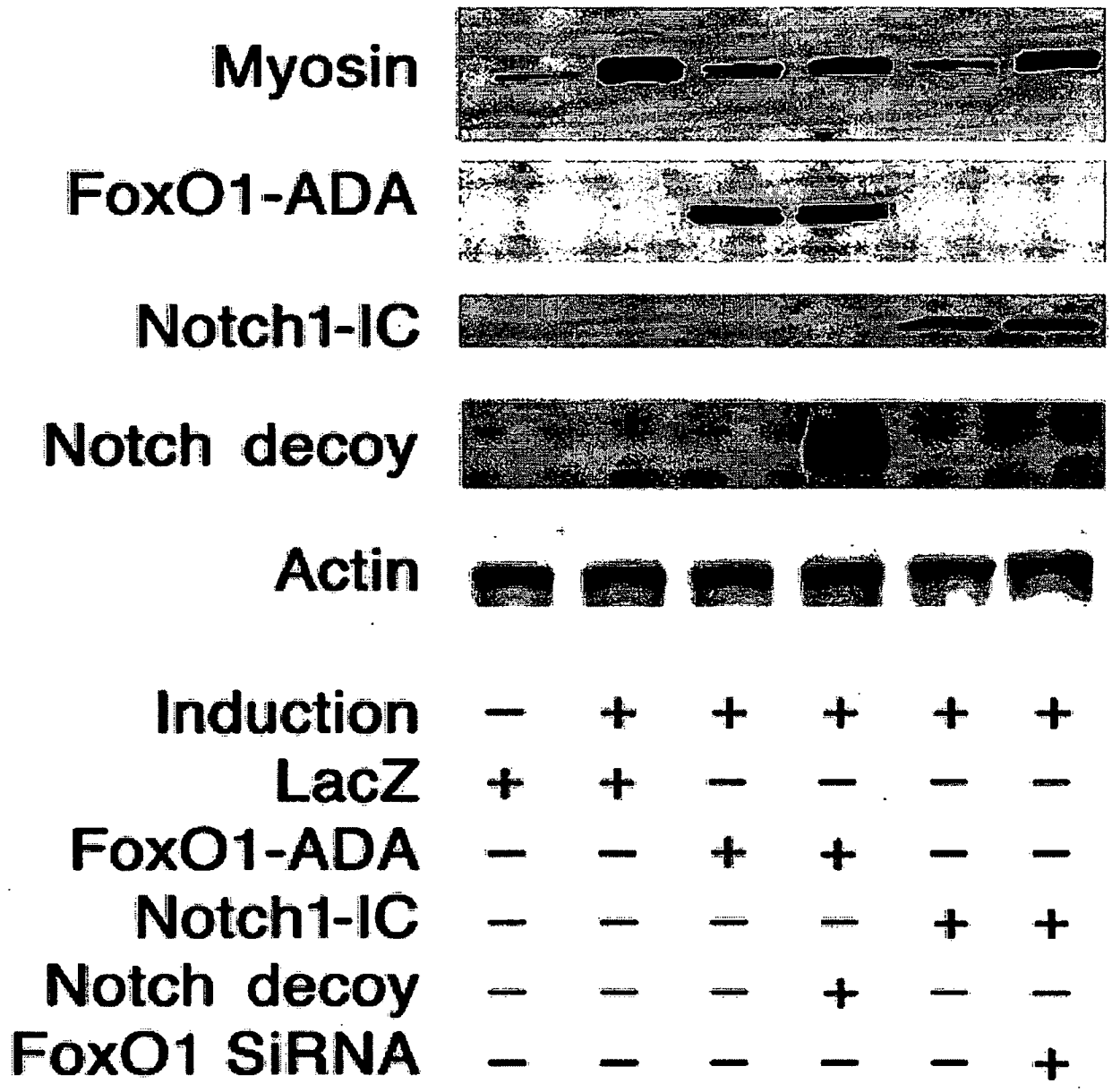

Myosin
FoxO1-ADA
Notch1-IC
Notch decoy
Actin
Induction − + + + + +
LacZ + + − − − −
FoxO1-ADA − − + + − −
Notch1-IC − − − − + +
Notch decoy − − − + − −
FoxO1 SiRNA − − − − − +

FIG. 1B

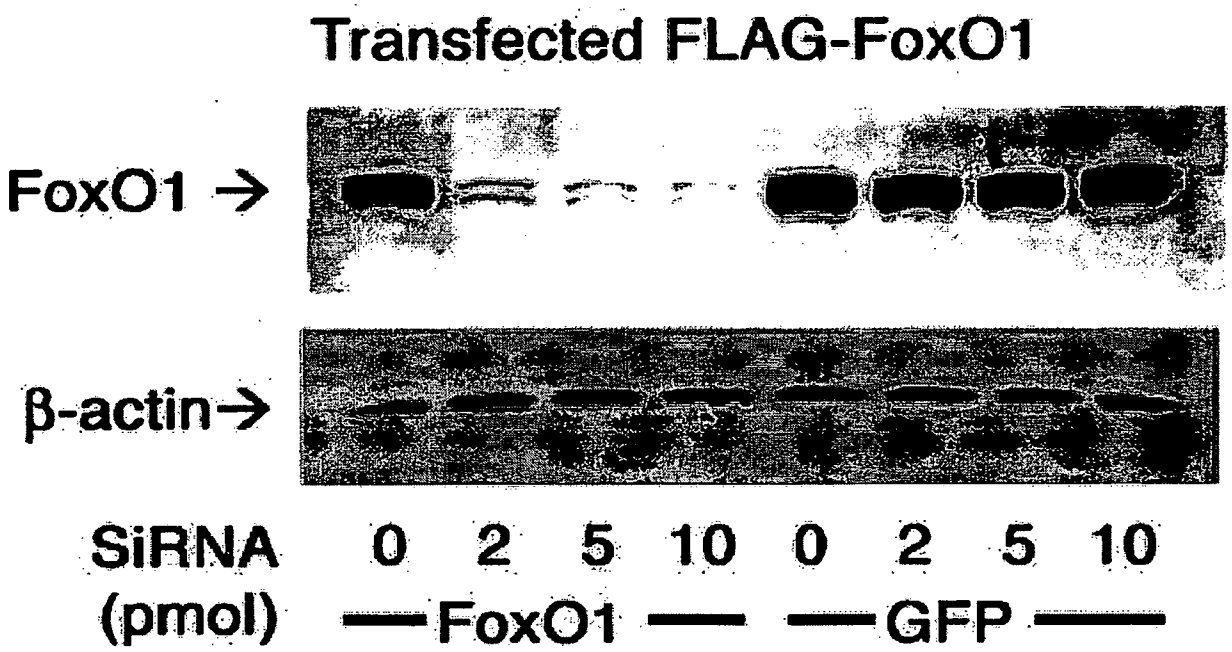

Transfected FLAG-FoxO1
FoxO1 →
β-actin→
SiRNA 0 2 5 10 0 2 5 10
(pmol) FoxO1 GFP

FIG. 1C

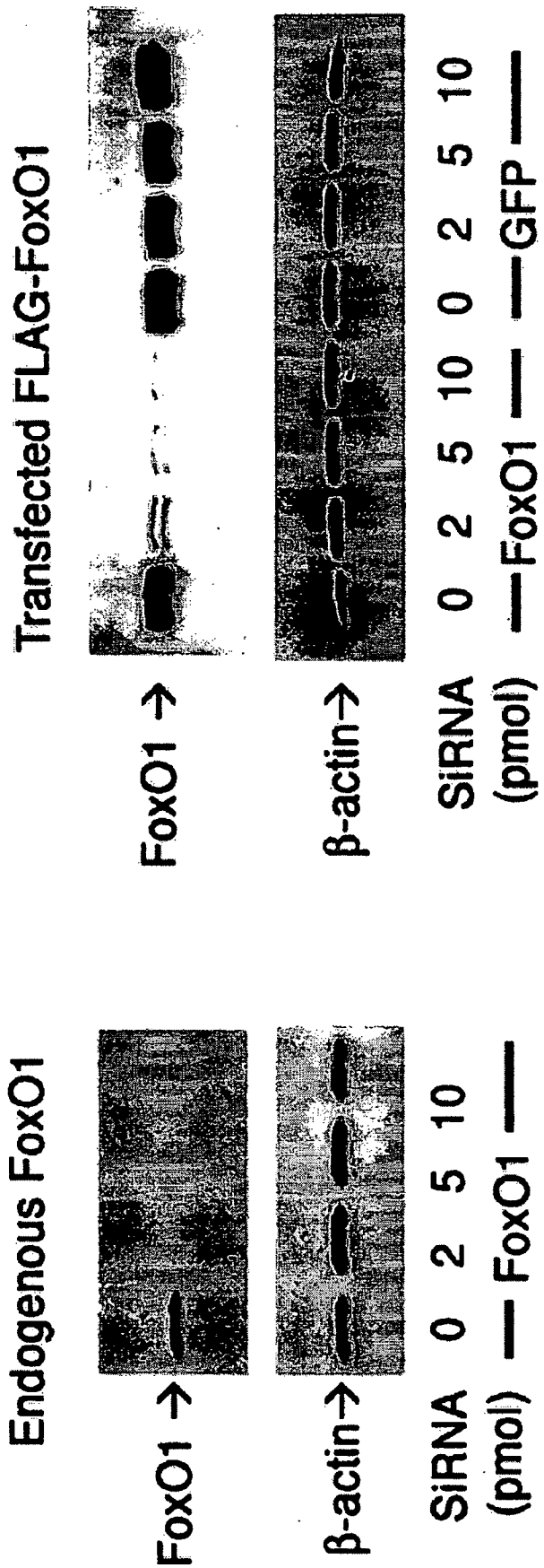

Endogenous FoxO1
FoxO1 →
β-actin→
SiRNA 0 2 5 10
(pmol) — FoxO1 —
Transfected FLAG-FoxO1
FoxO1 →
β-actin→
SiRNA 0 2 5 10 0 2 5 10
(pmol) — FoxO1 — — GFP —

FIG. 1D

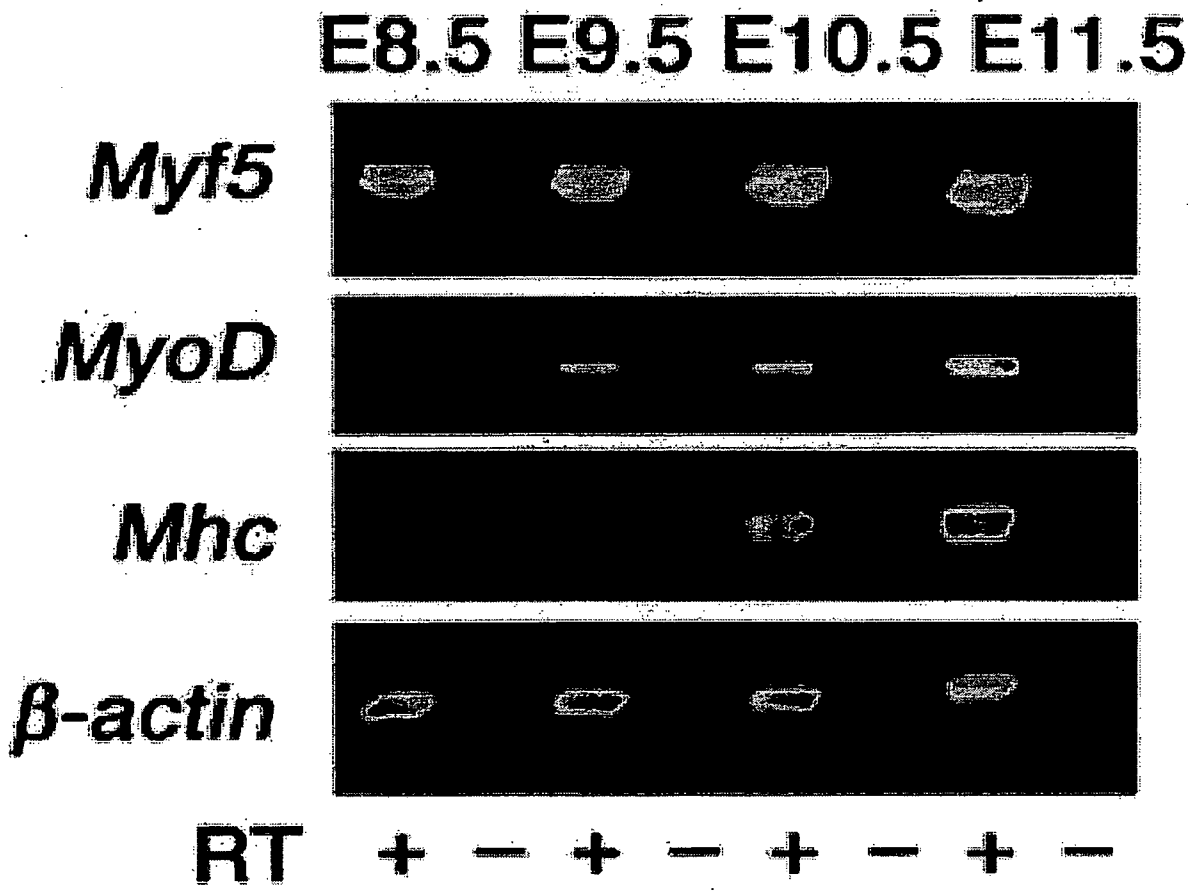


FIG. 1E

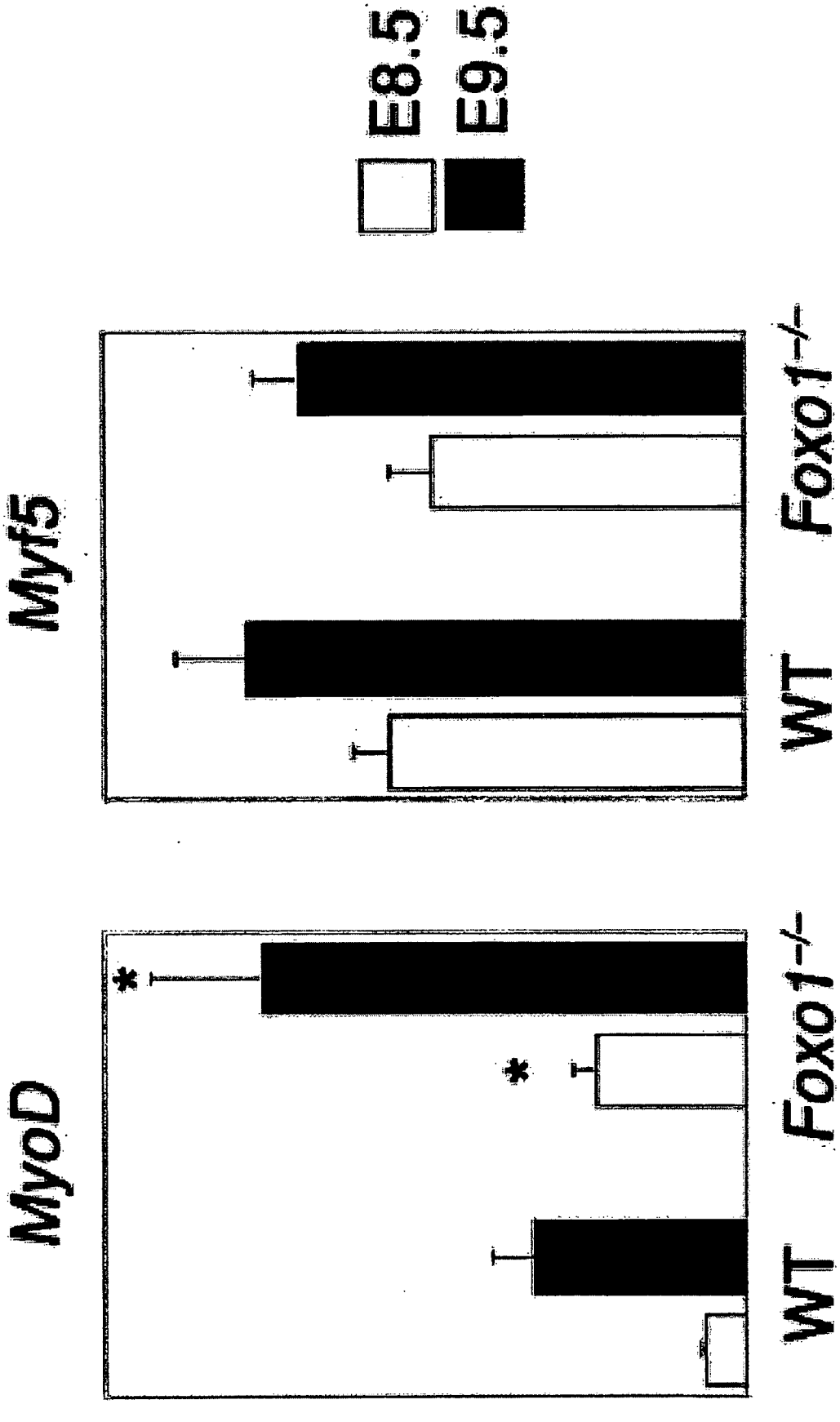


FIG. 1F

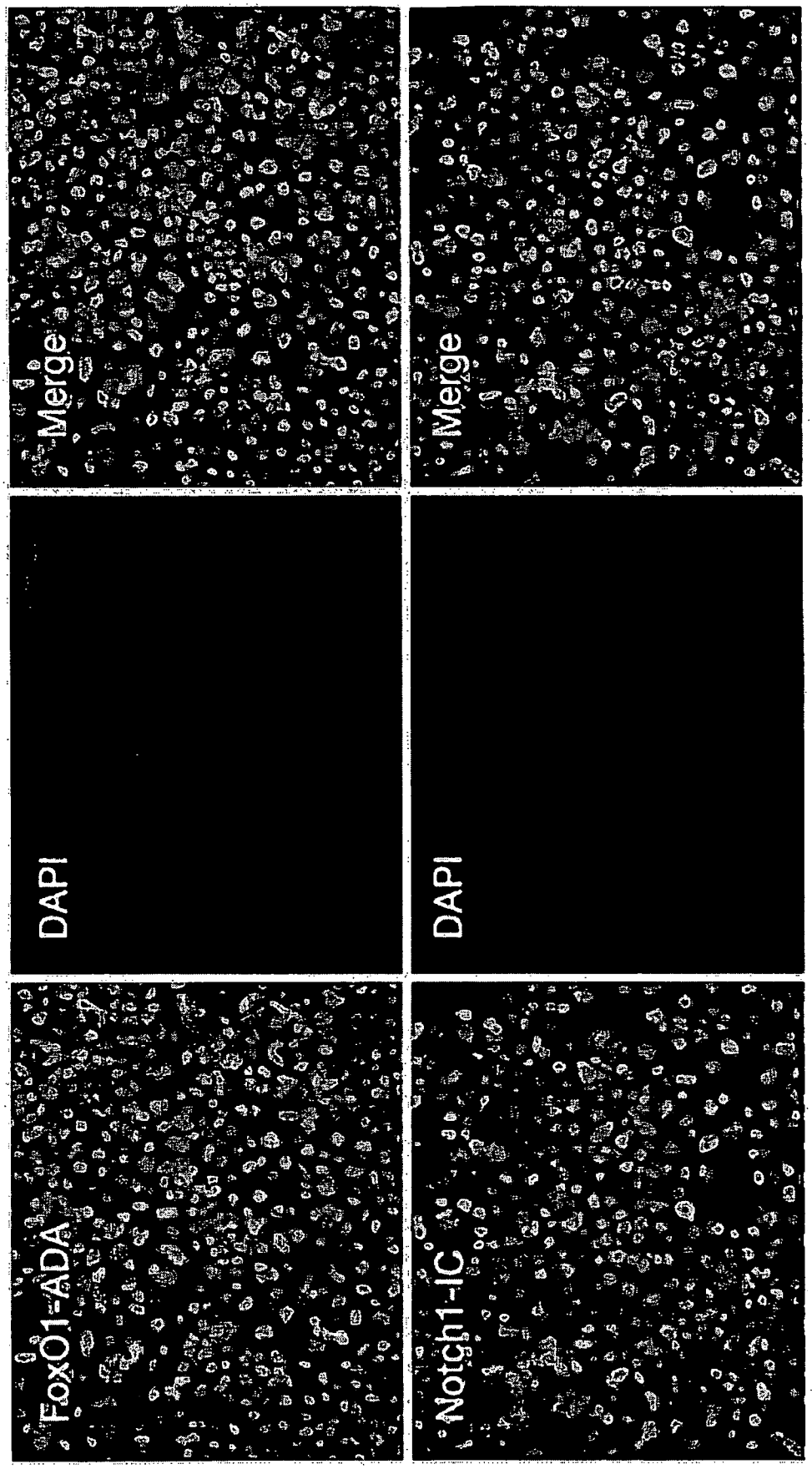

Adenoviral Transduction Efficiency
FoxO1-ADA
DAPI
Merge
Notch1-IC
DAPI
Merge

FIG. 1G

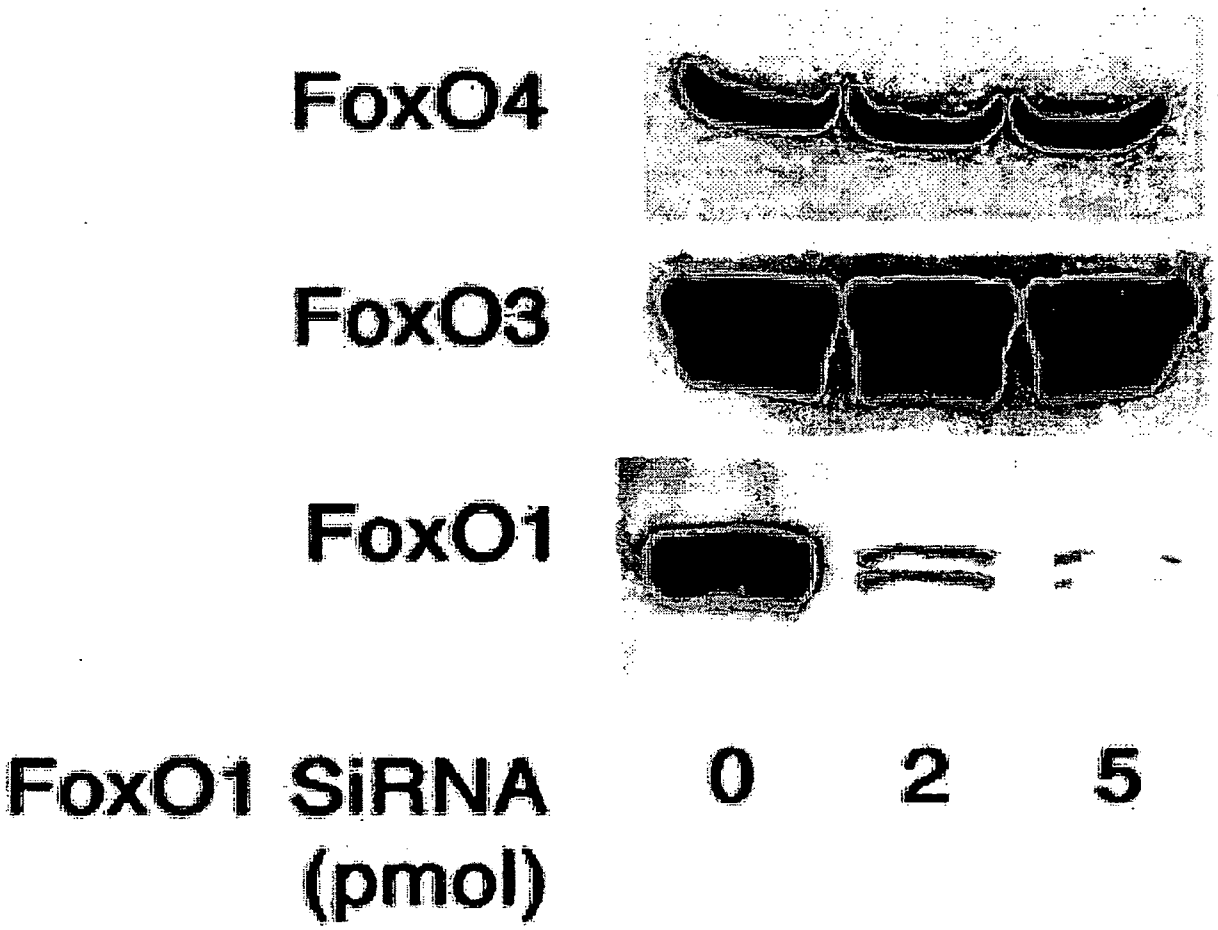


FIG. 1H

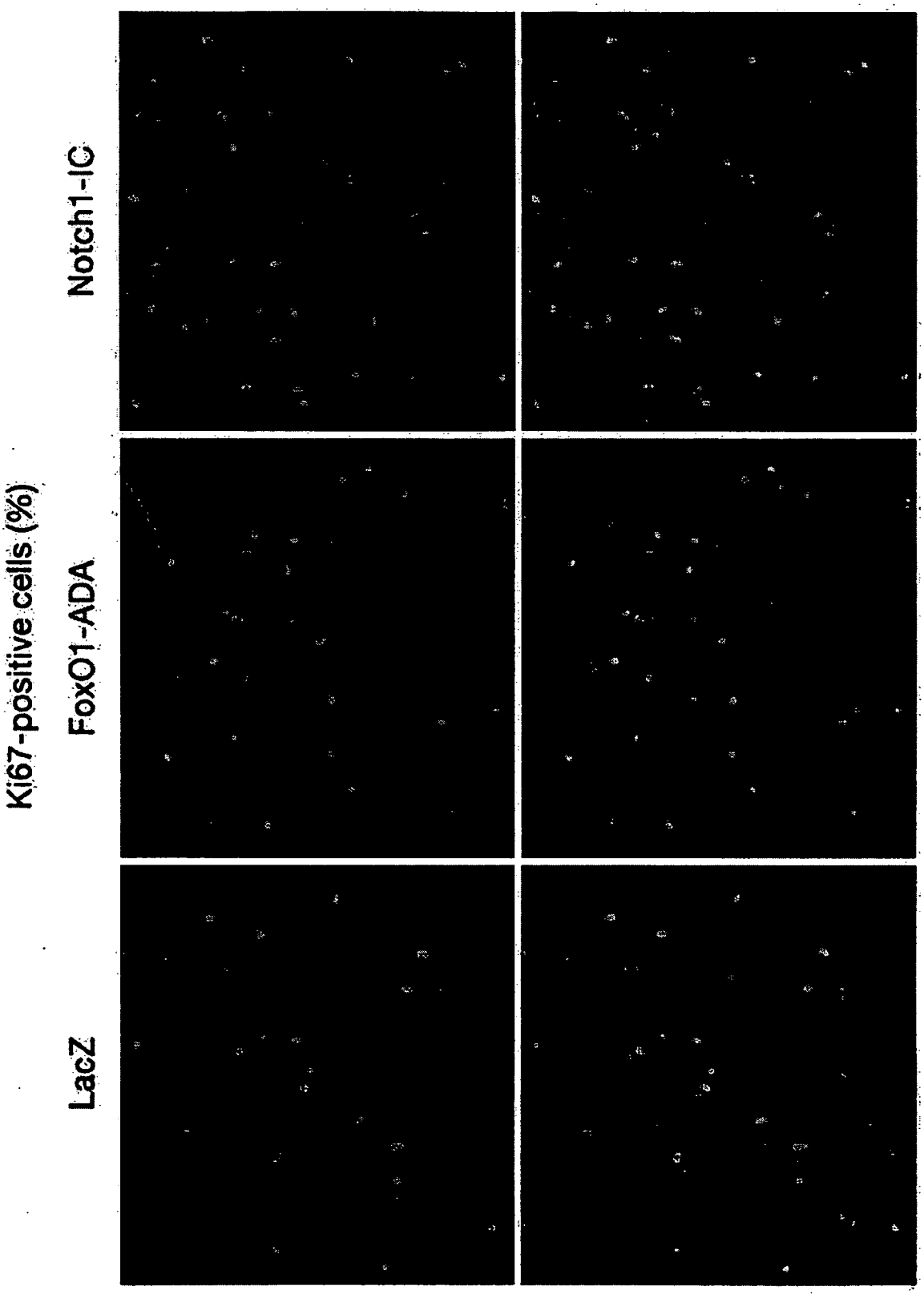


FIG. 1I

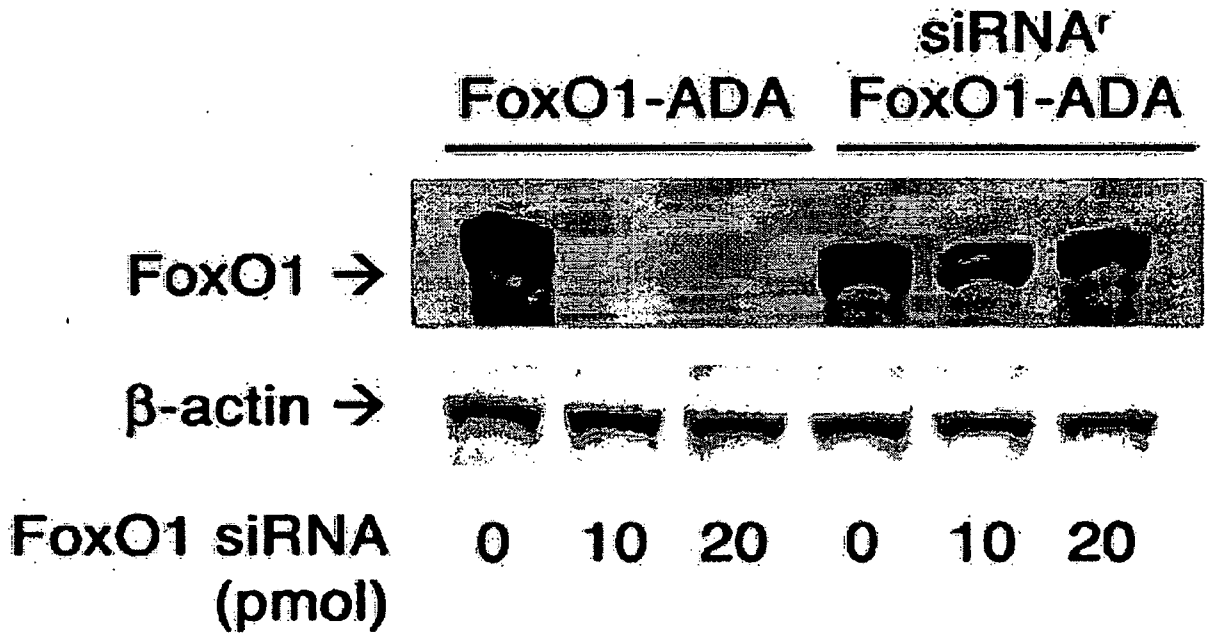

siRNA
FoxO1-ADA
FoxO1-ADA
FoxO1 →
β-actin →
FoxO1 siRNA (pmol)
0
10
20
0
10
20

FIG. 1J

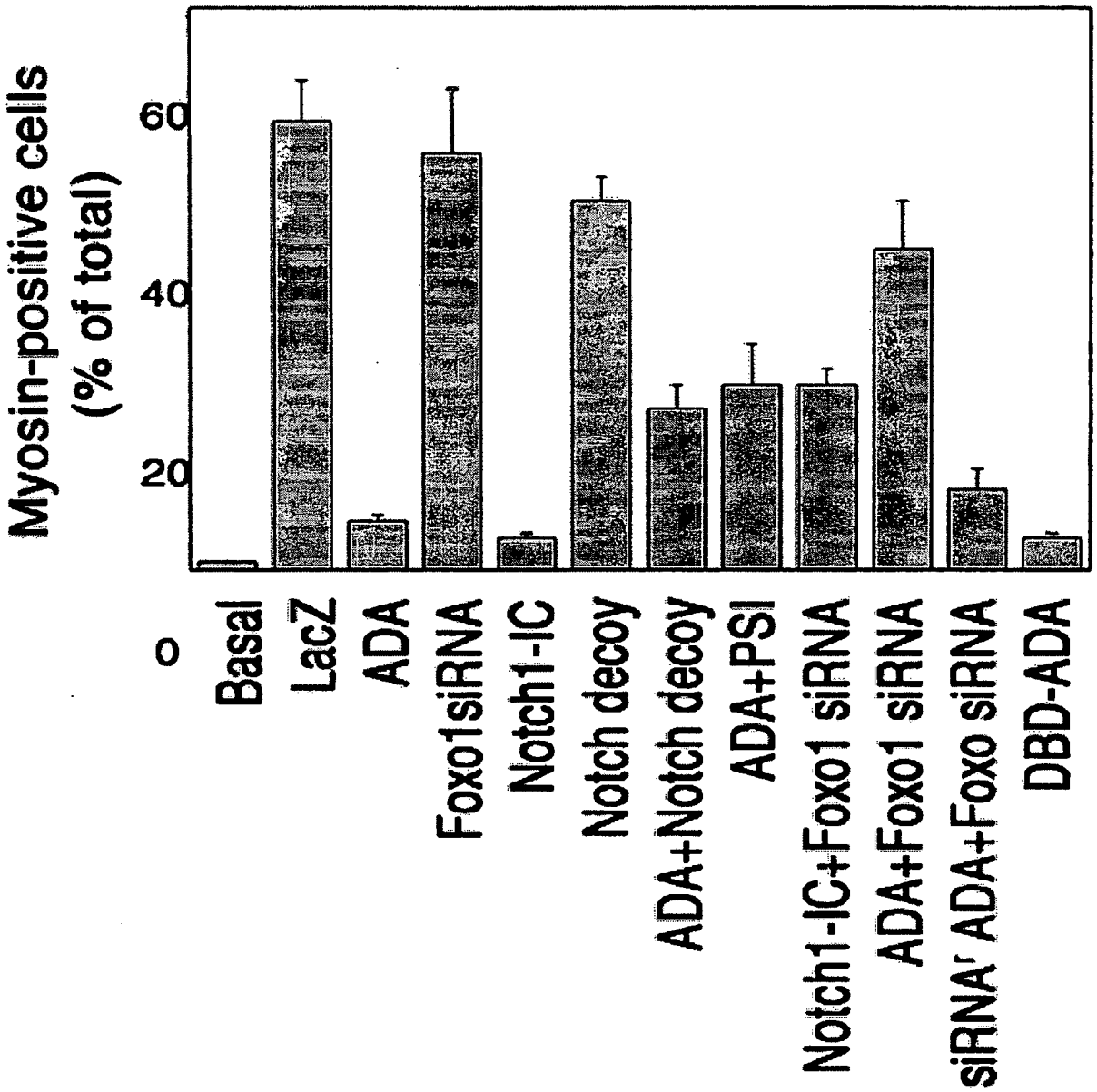

Myosin-positive cells
(% of total)
0
20
40
60
Basal
LacZ
ADA
Foxo1siRNA
Notch1-IC
Notch decoy
ADA+Notch decoy
ADA+PSI
Notch1-IC+Foxo1 siRNA
ADA+Foxo1 siRNA
siRNA' ADA+Foxo siRNA
DBD-ADA

FIG. 1K

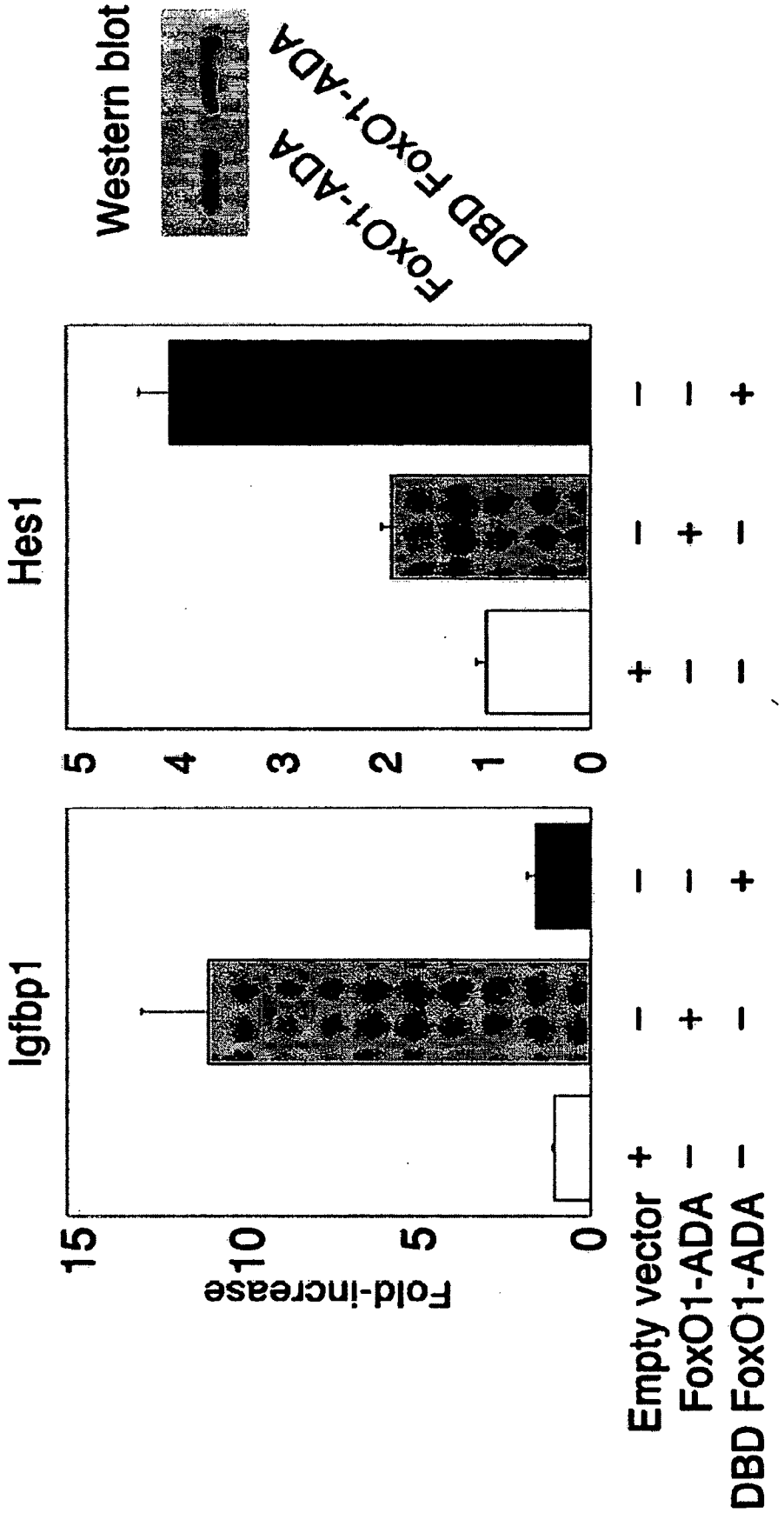

Western blot
FoxO1-ADA
DBD FoxO1-ADA
Hes1
Igfbp1
Fold-increase
0
1
2
3
4
5
0
5
10
15
Empty vector + - -
FoxO1-ADA - + -
DBD FoxO1-ADA - - +

FIG. 1L

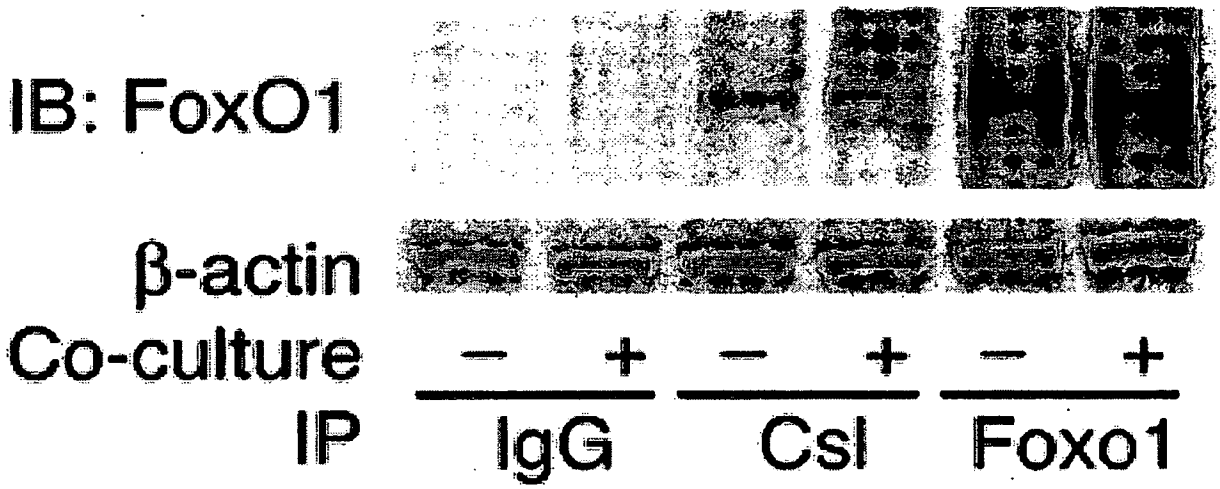


FIG. 2A

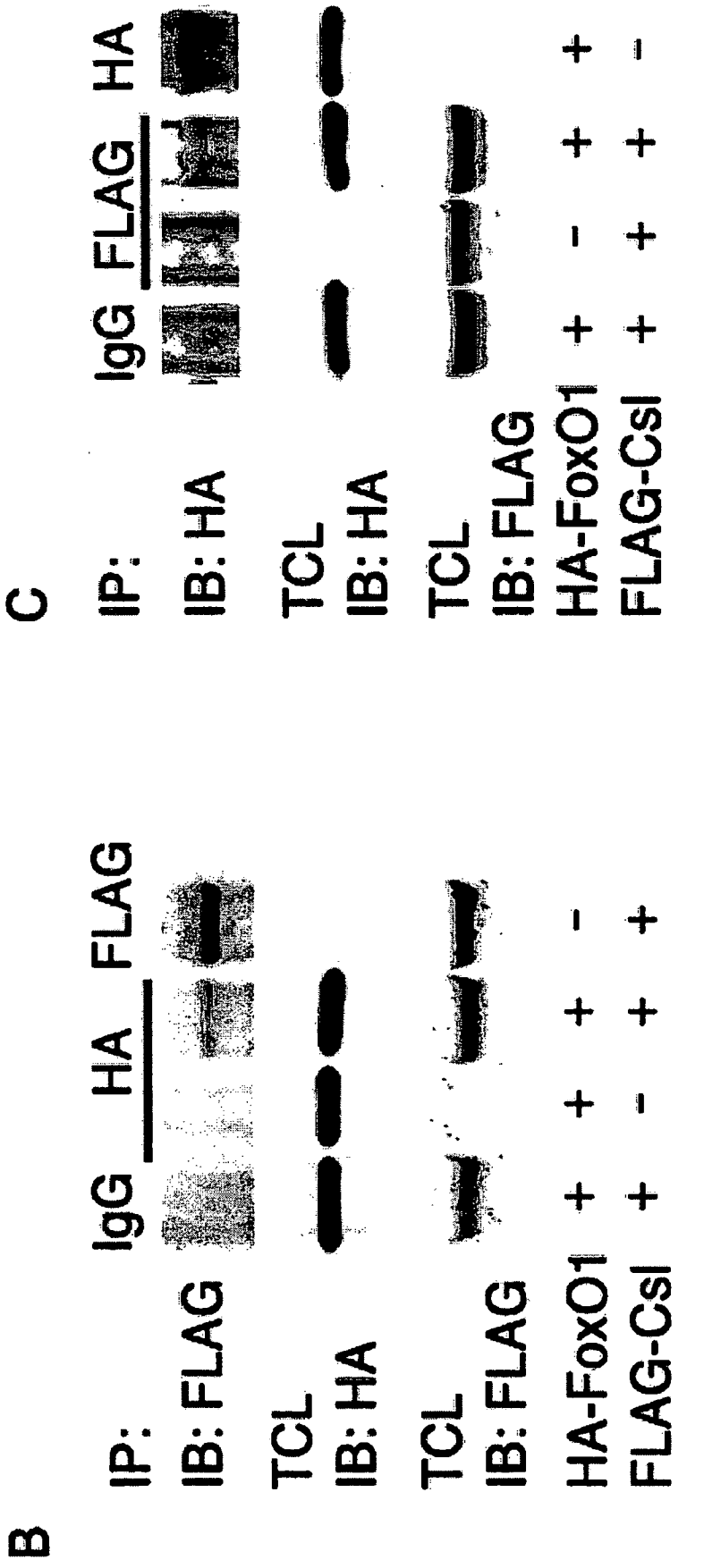

B
IP:
IgG
HA
FLAG
IB: FLAG
TCL
IB: HA
TCL
IB: FLAG
HA-FoxO1 + + -
FLAG-CsI + - +
C
IP:
IgG
FLAG
HA
IB: HA
TCL
IB: HA
TCL
IB: FLAG
HA-FoxO1 + - + +
FLAG-CsI + + + -

FIG. 2B-C

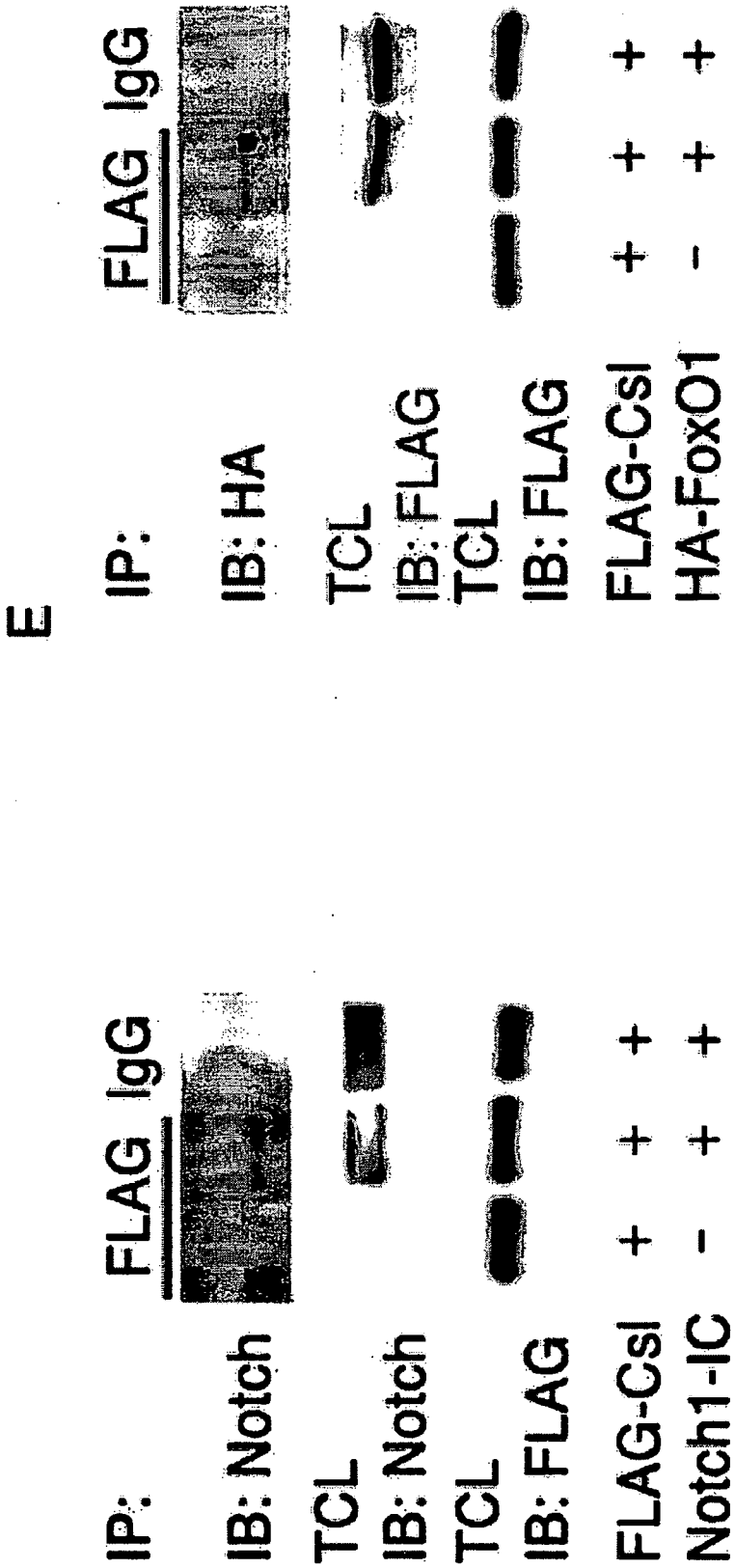

D
IP:
FLAG IgG
IB: Notch
TCL
IB: Notch
TCL
IB: FLAG
FLAG-CsI + + +
Notch1-IC - + +
E
IP:
FLAG IgG
IB: HA
TCL
IB: FLAG
TCL
IB: FLAG
FLAG-CsI + + +
HA-FoxO1 - + +

FIG. 2D-E

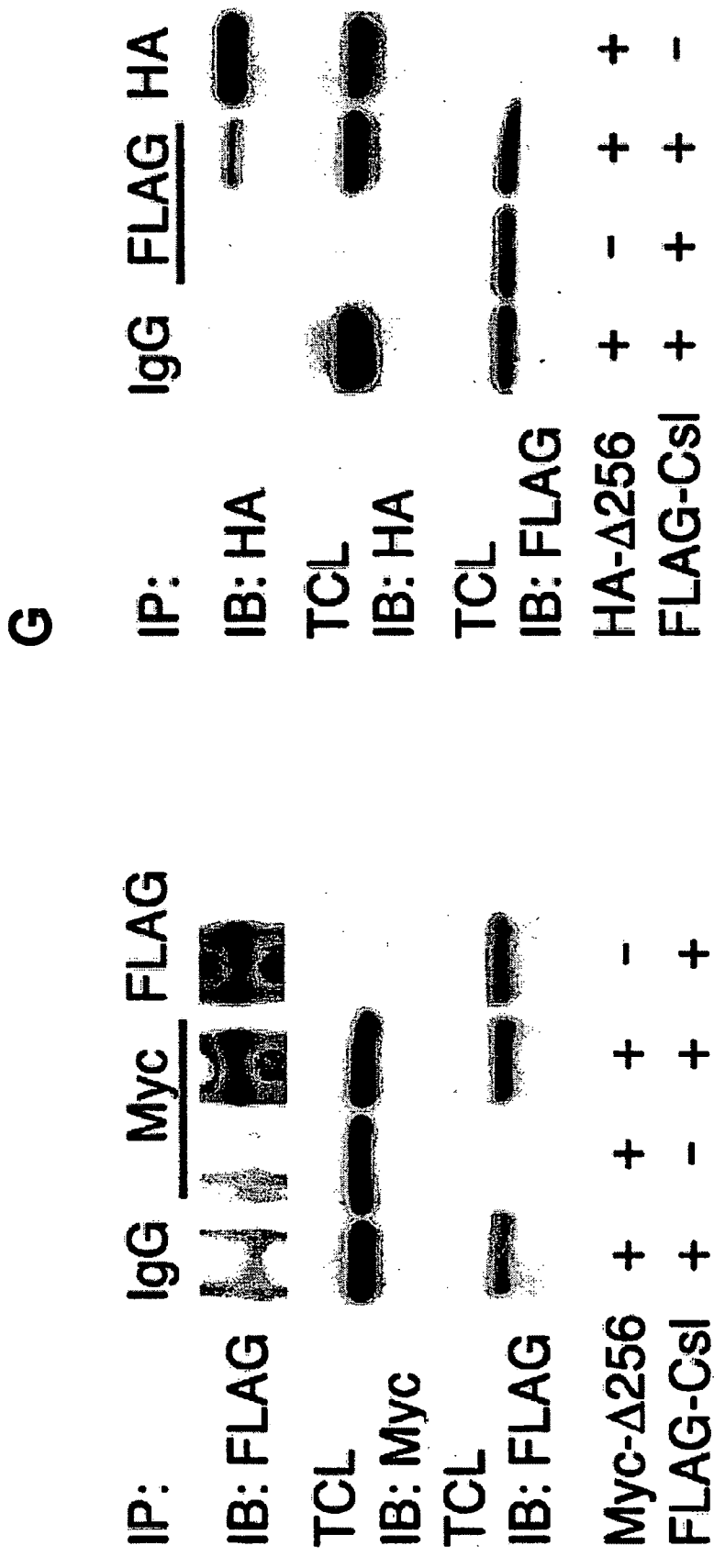

F
IP:
IgG
Myc
FLAG
IB: FLAG
TCL
IB: Myc
TCL
IB: FLAG
Myc-Δ256 + + + -
FLAG-CsI + - + +
G
IP:
IgG
FLAG
HA
IB: HA
TCL
IB: HA
TCL
IB: FLAG
HA-Δ256 + - + +
FLAG-CsI + + + -

FIG. 2F- 2G

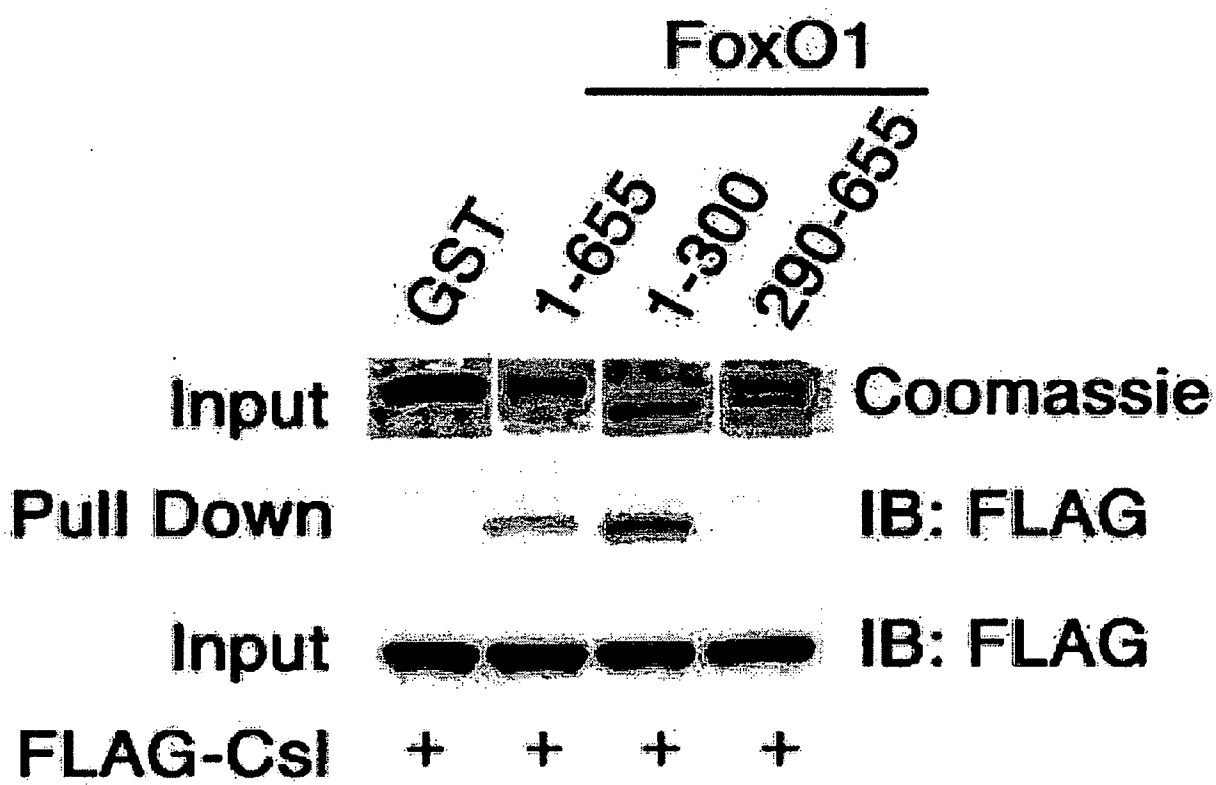

FoxO1
GST
1-655
1-300
290-655
Input
Coomassie
Pull Down
IB: FLAG
Input
IB: FLAG
FLAG-Csl
+ + + +

FIG. 2H

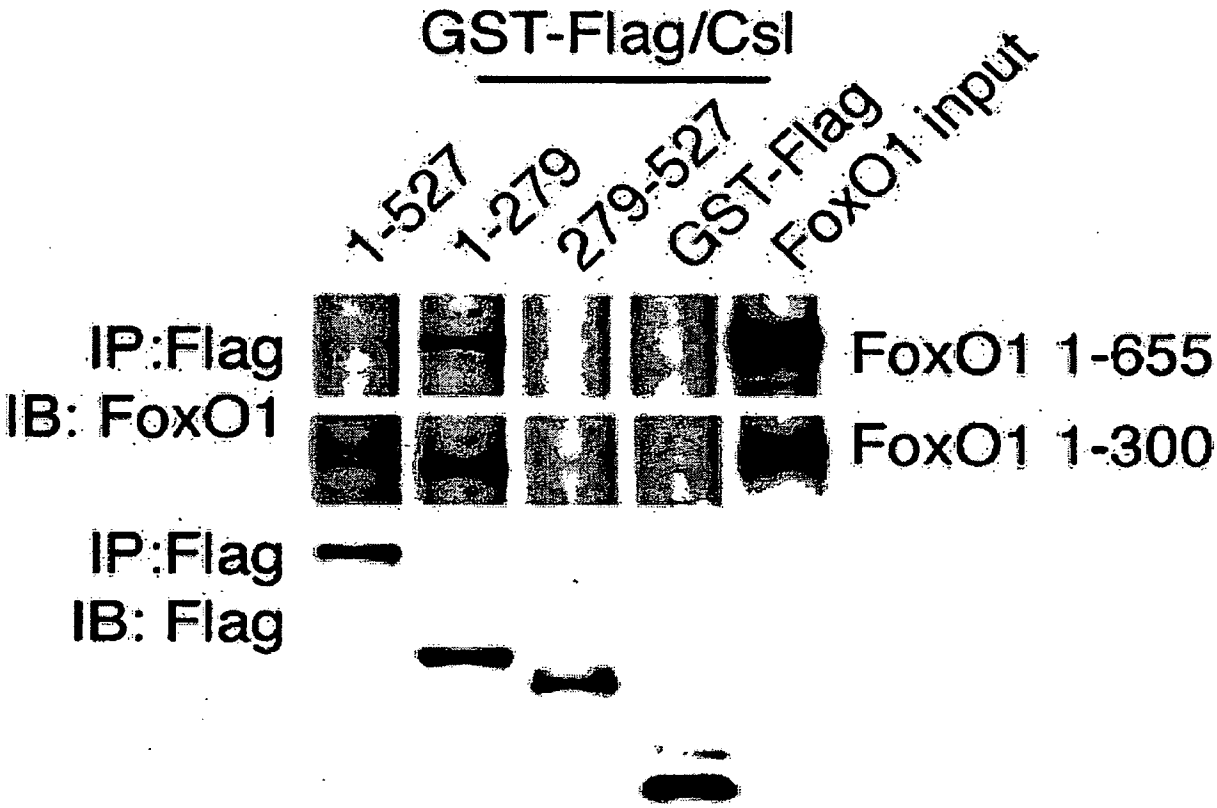

GST-Flag/Csl
1-527
1-279
279-527
GST-Flag
FoxO1 input
IP:Flag
IB: FoxO1
FoxO1 1-655
FoxO1 1-300
IP:Flag
IB: Flag

FIG. 2I

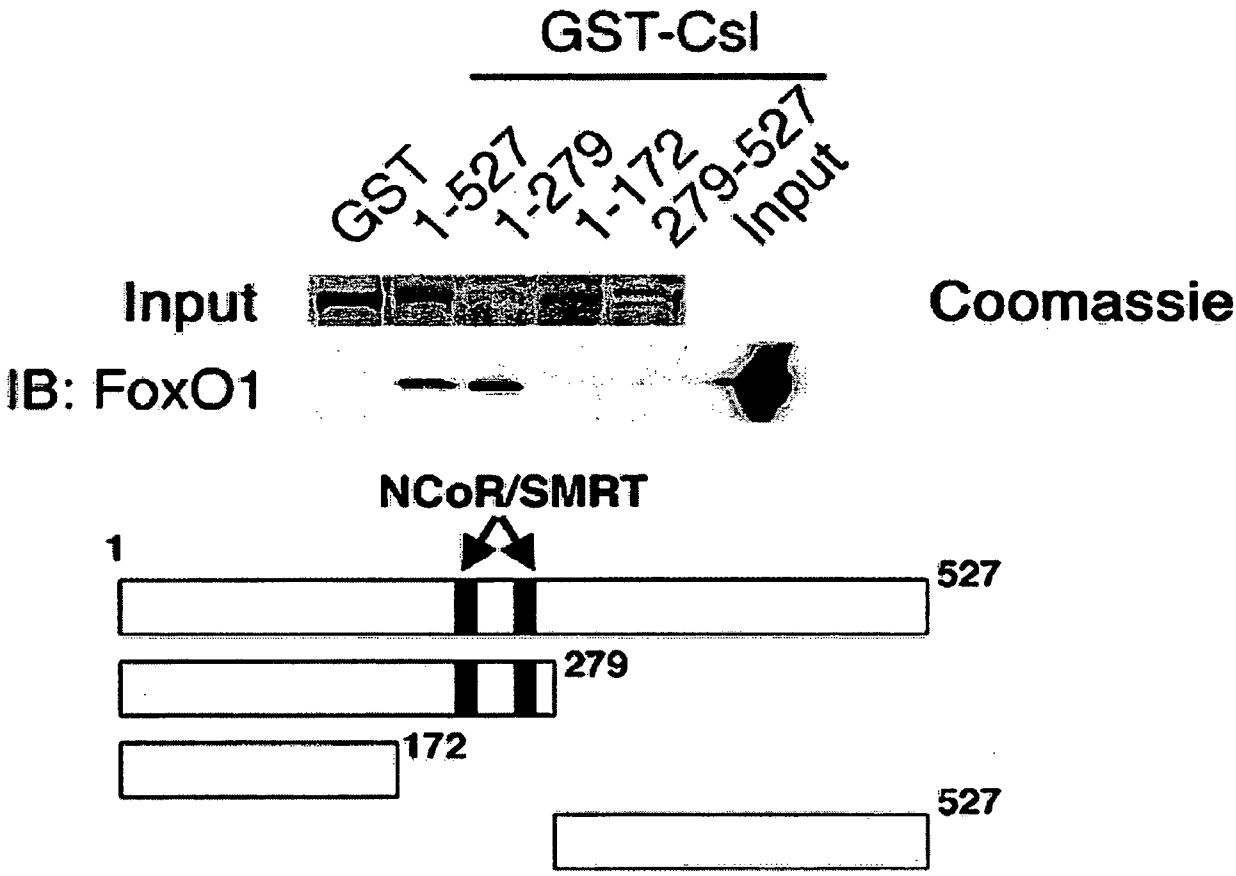

GST-Csl
GST
1-527
1-279
1-172
279-527
Input
Input
Coomassie
IB: FoxO1
NCoR/SMRT
1
527
279
172
527

FIG. 2J

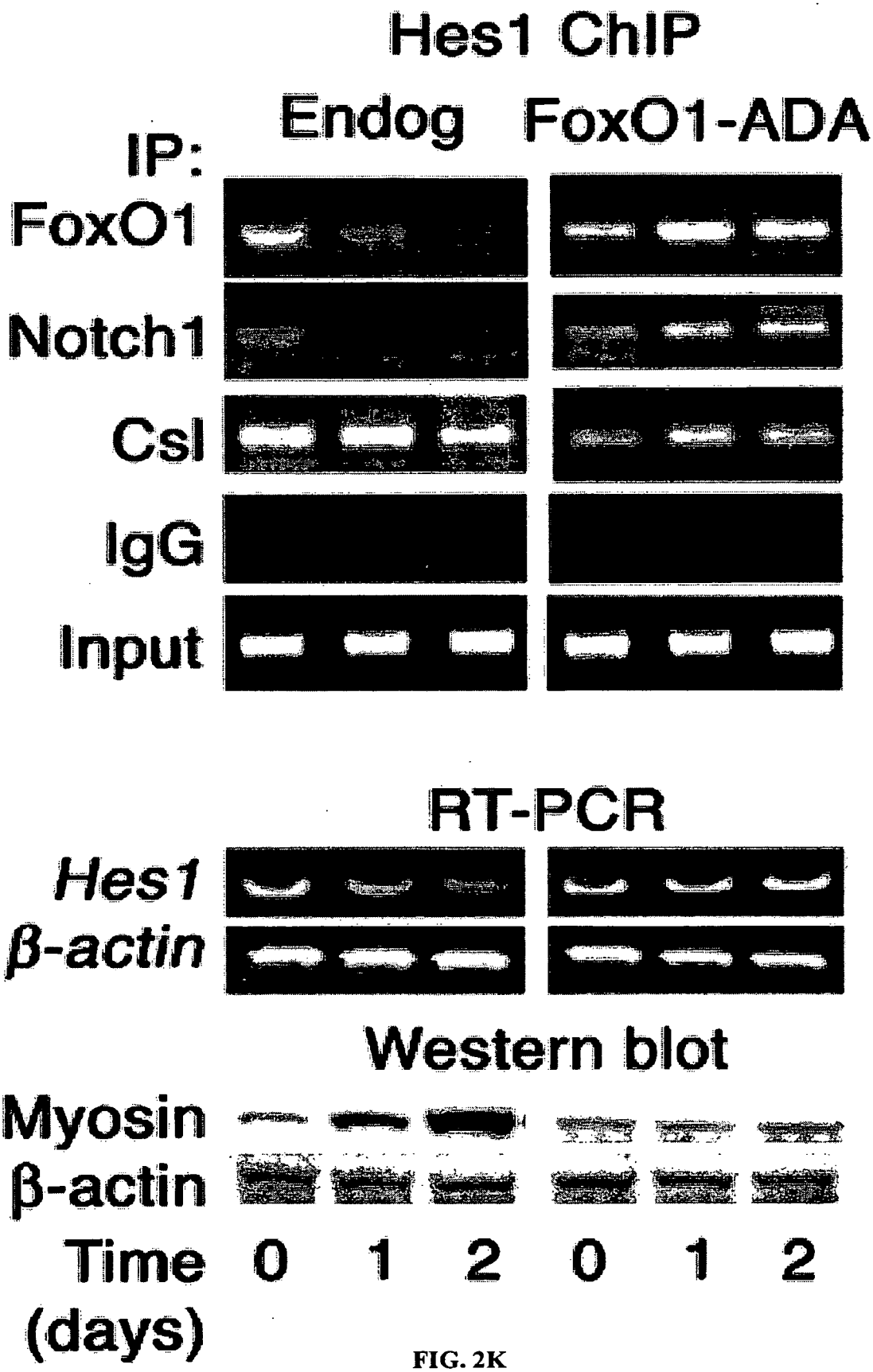

Hes1 ChIP
Endog
FoxO1-ADA
IP:
FoxO1
Notch1
Csl
IgG
Input
RT-PCR
Hes1
β-actin
Western blot
Myosin
β-actin
Time
(days)
0
1
2
0
1
2

FIG. 2K

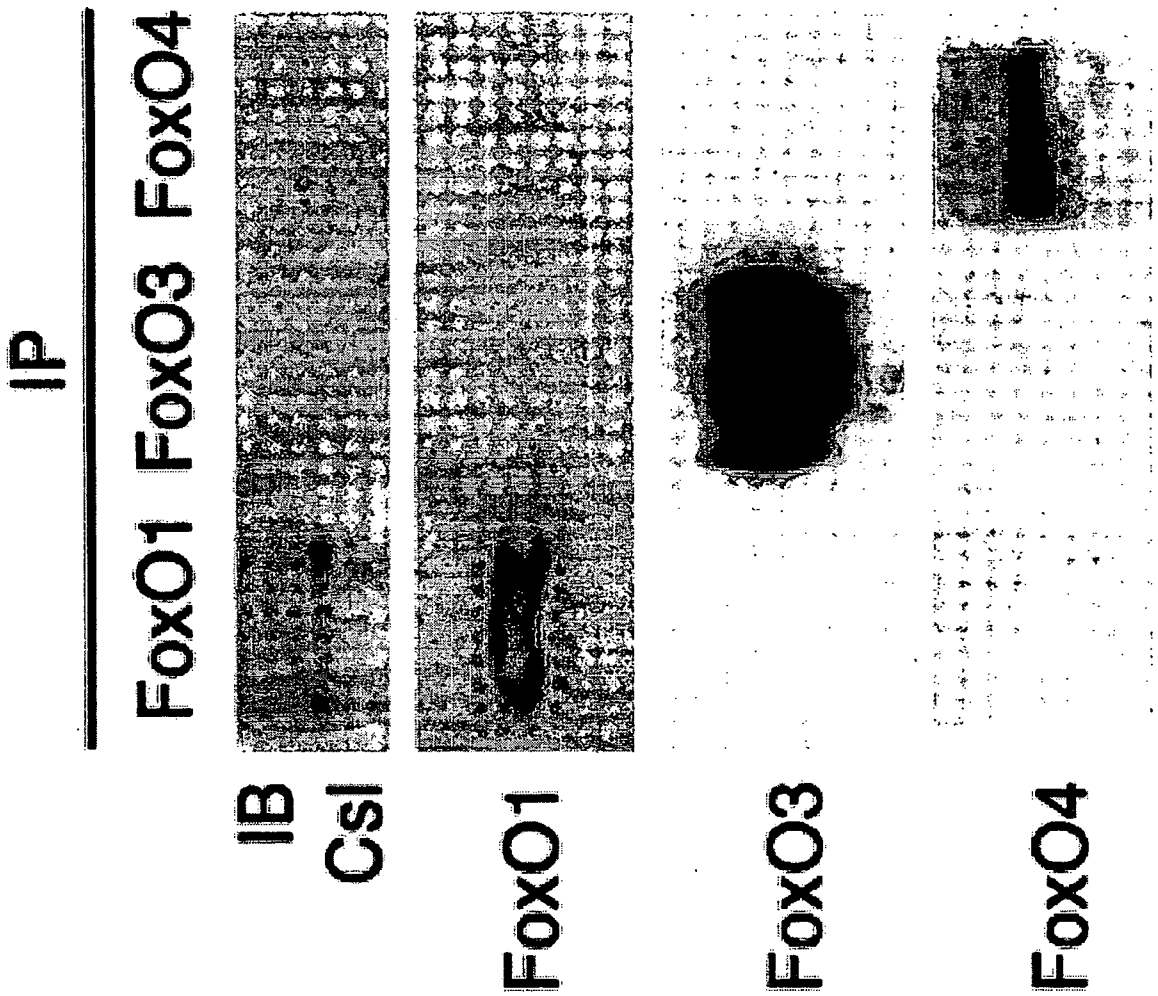


FIG. 2L

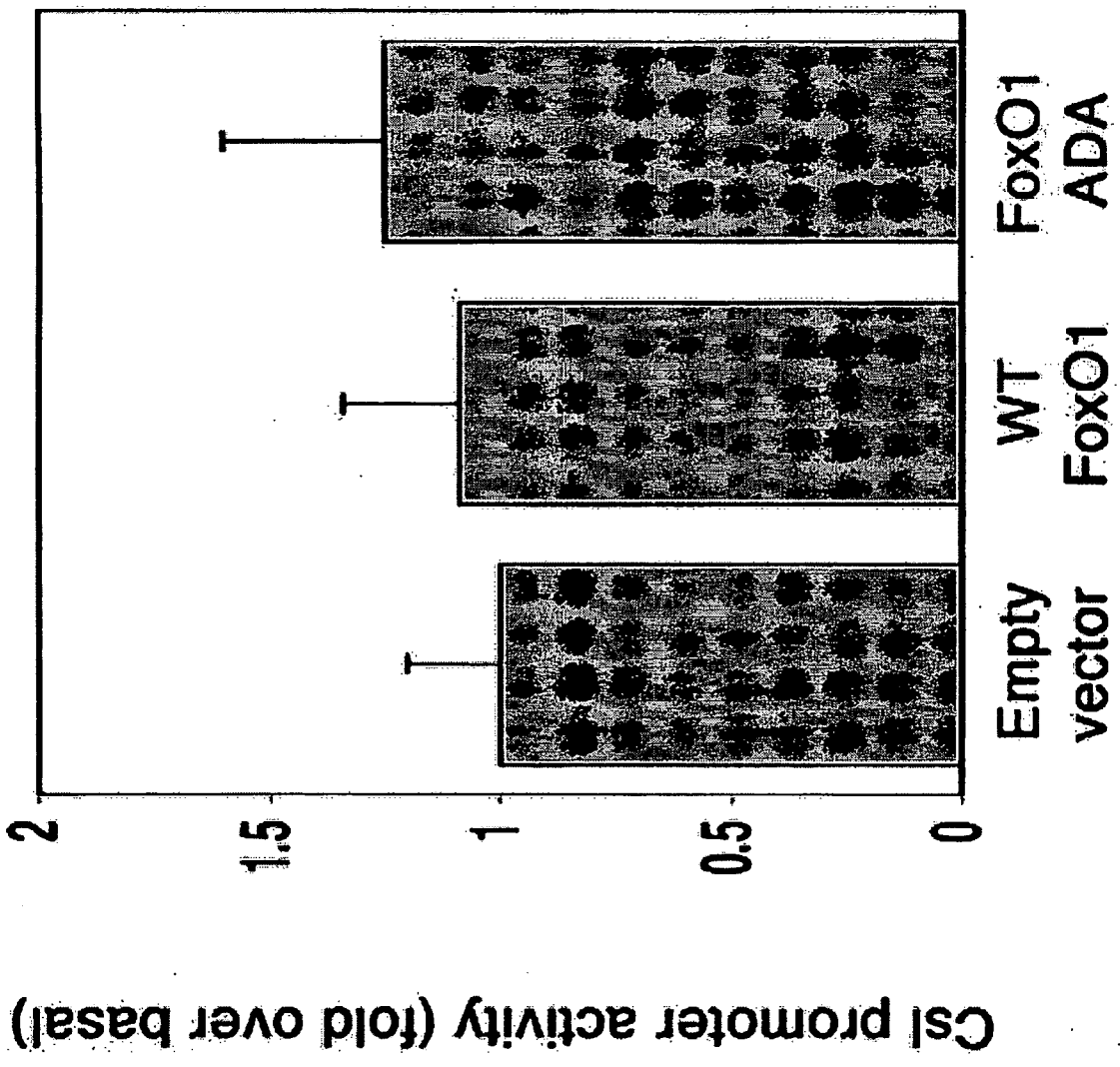

Csl promoter activity (fold over basal)
2
1.5
1
0.5
0
Empty vector
WT FoxO1
FoxO1 ADA

FIG. 2M

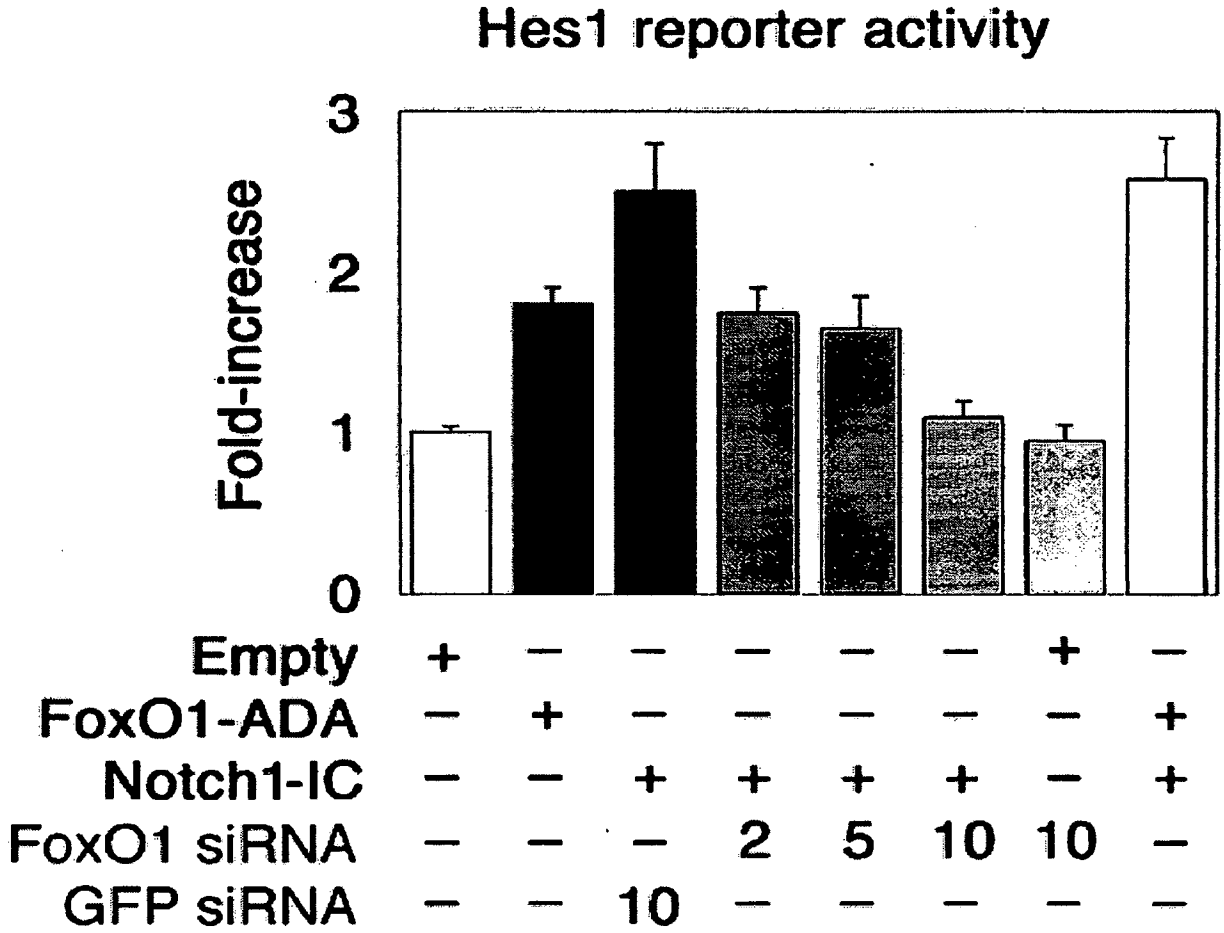

Hes1 reporter activity
Fold-increase
3
2
1
0
Empty + – – – – – + –
FoxO1-ADA – + – – – – – +
Notch1-IC – – + + + + – +
FoxO1 siRNA – – – 2 5 10 10 –
GFP siRNA – – 10 – – – – –

FIG. 3A

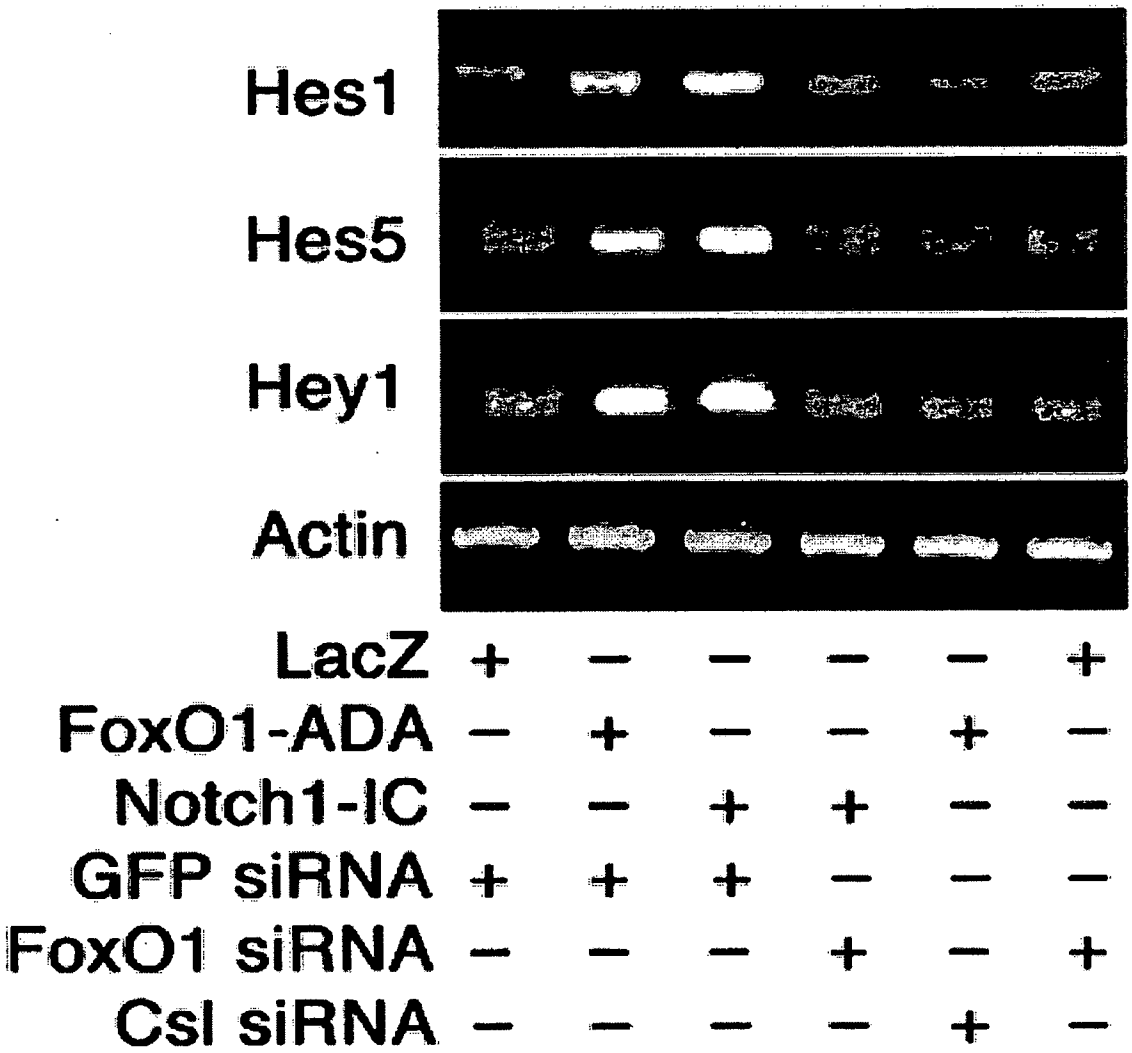

Hes1
Hes5
Hey1
Actin
LacZ + − − − − +
FoxO1-ADA − + − − + −
Notch1-IC − − + + − −
GFP siRNA + + + − − −
FoxO1 siRNA − − − + − +
Csl siRNA − − − − + −

FIG. 3B

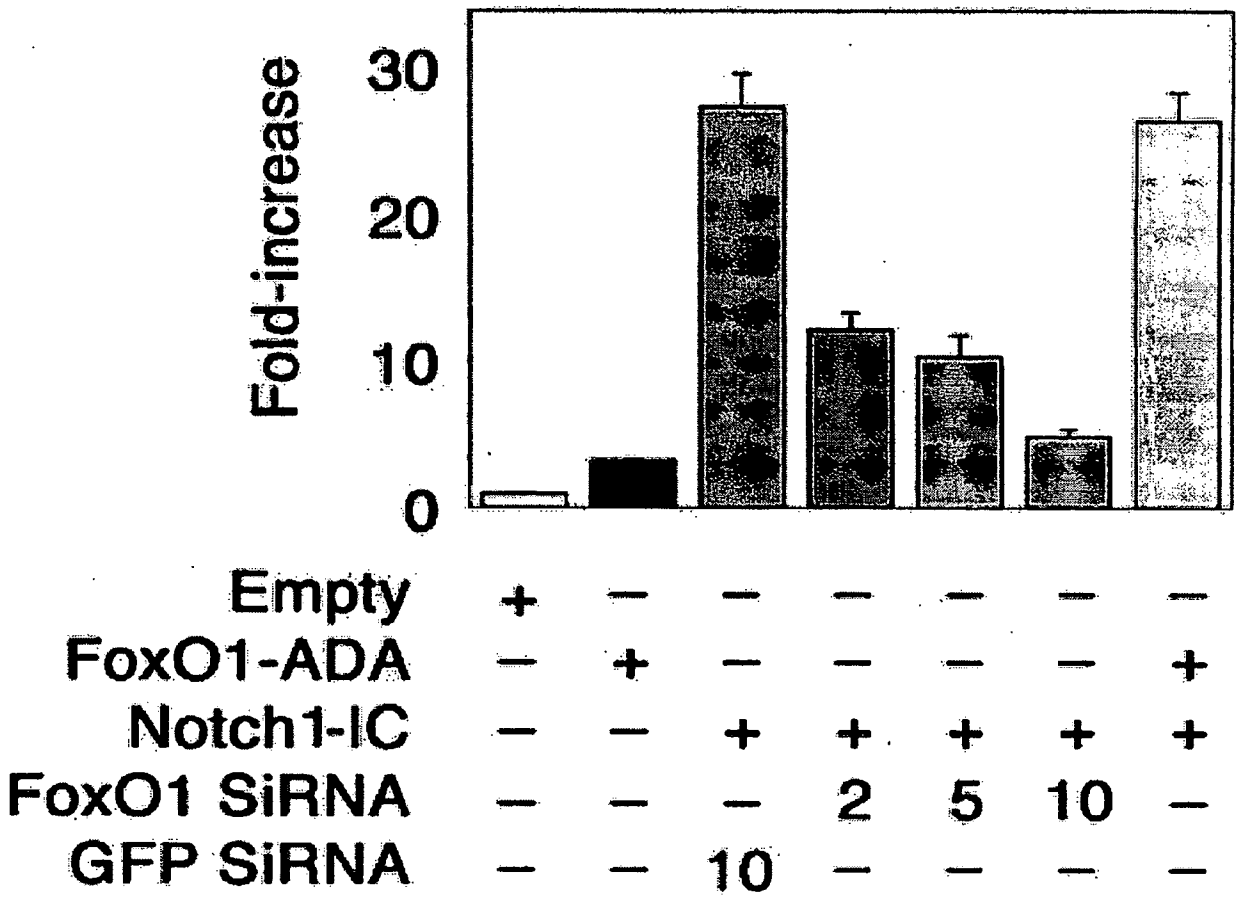

Fold-increase
30
20
10
0
Empty + – – – – – –
FoxO1-ADA – + – – – – +
Notch1-IC – – + + + + +
FoxO1 SiRNA – – – 2 5 10 –
GFP SiRNA – – 10 – – – –

FIG. 3C

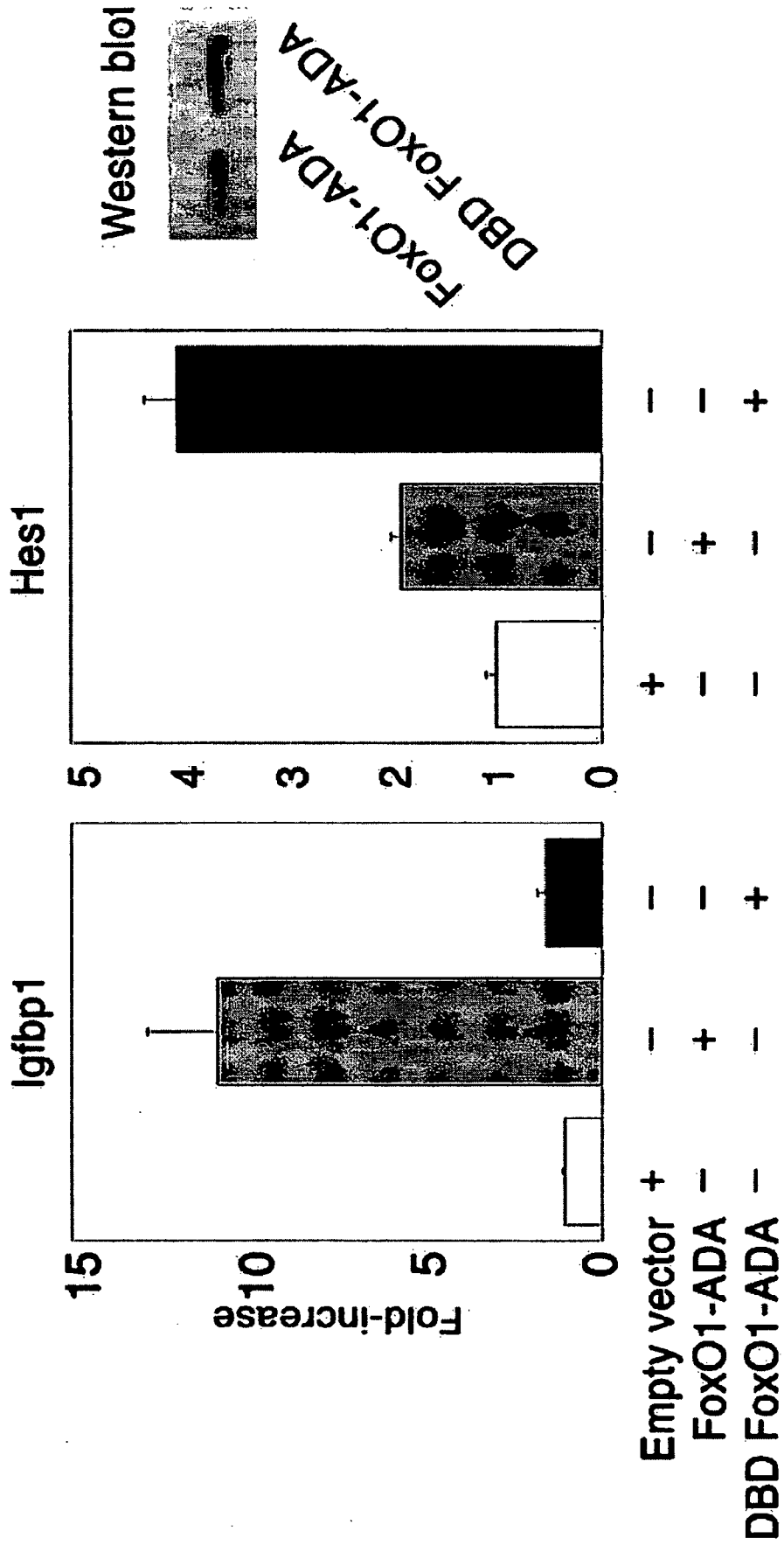
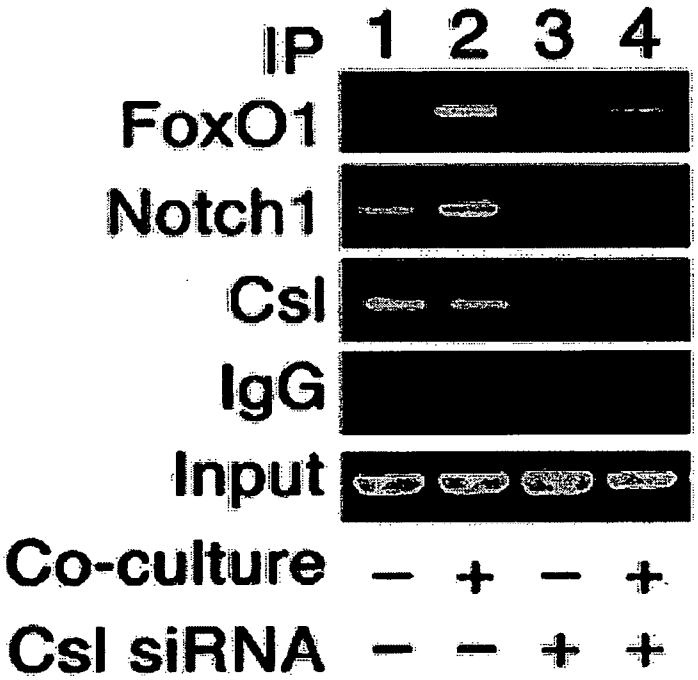

Western blot
FoxO1-ADA
DBD FoxO1-ADA
Hes1
Igfbp1
Fold-increase
0
5
10
15
1
2
3
4
Empty vector
FoxO1-ADA
DBD FoxO1-ADA

FIG. 3D

A


IP 1 2 3 4
FoxO1
Notch1
Csl
IgG
Input
Co-culture – + – +
Csl siRNA – – + +

B

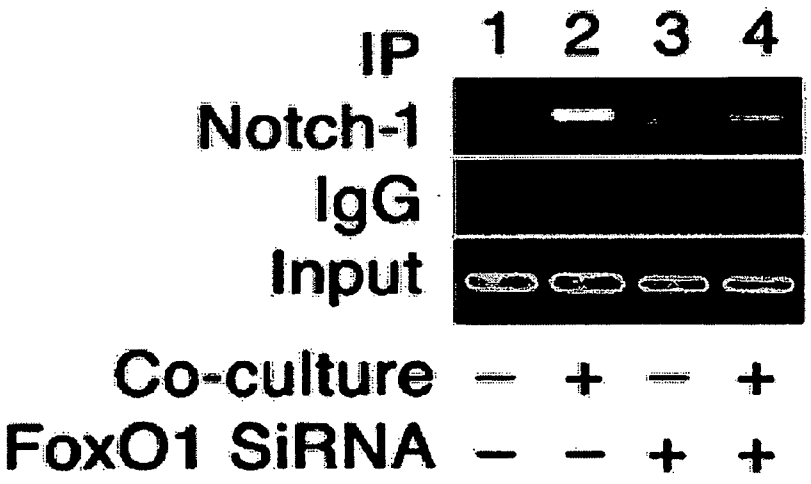

IP 1 2 3 4
Notch-1
IgG
Input
Co-culture – + – +
FoxO1 SiRNA – – + +

FIG. 4A-B

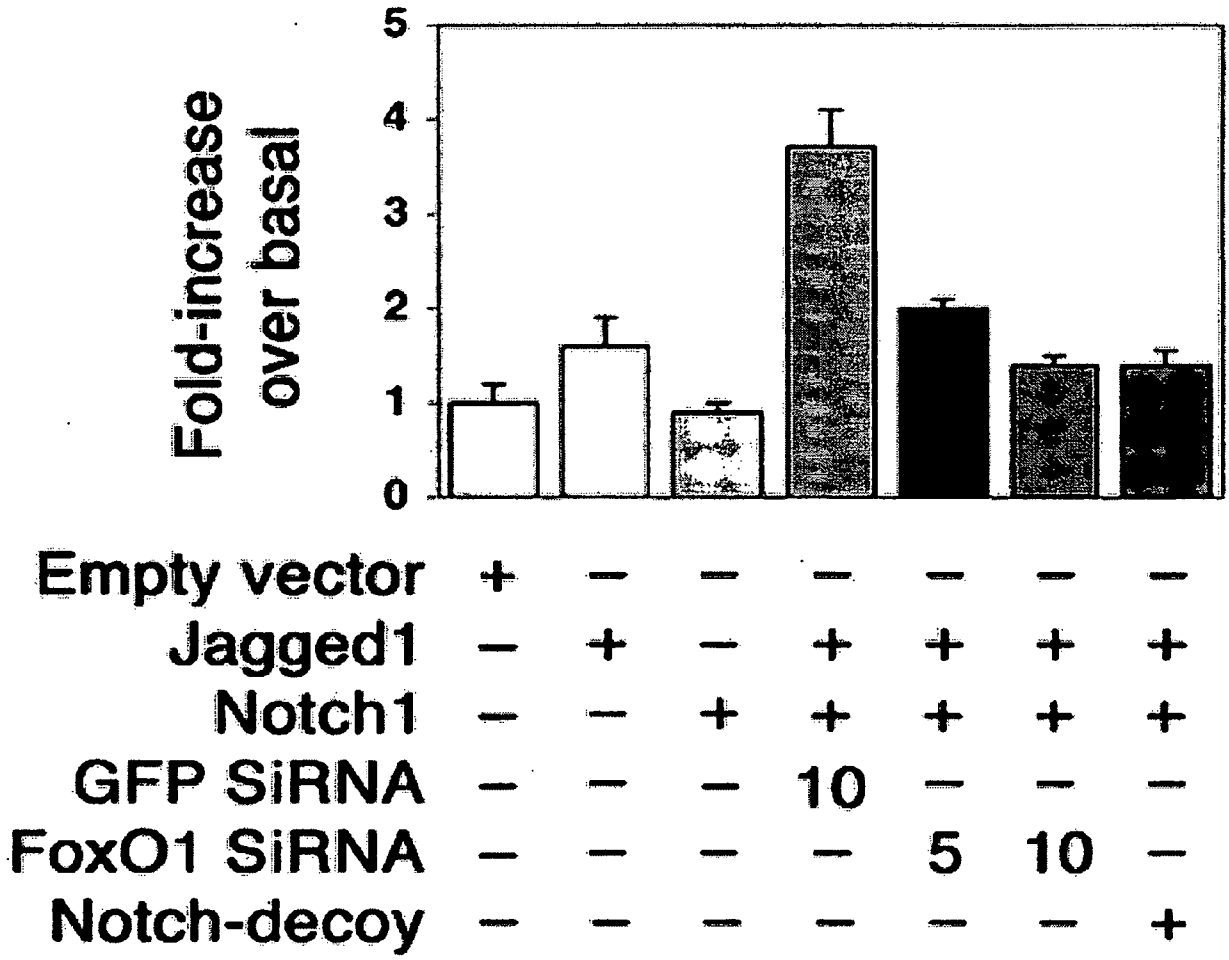
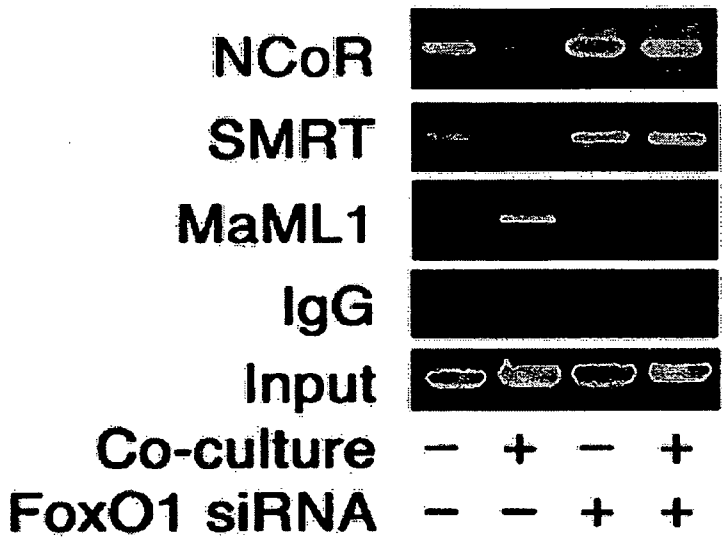

Fold-increase over basal
5
4
3
2
1
0
Empty vector + – – – – – –
Jagged1 – + – + + + +
Notch1 – – + + + + +
GFP SiRNA – – – 10 – – –
FoxO1 SiRNA – – – – 5 10 –
Notch-decoy – – – – – – +

FIG. 4C

D


IP
NCoR
SMRT
MaML1
IgG
Input
Co-culture − + − +
FoxO1 siRNA − − + +

E

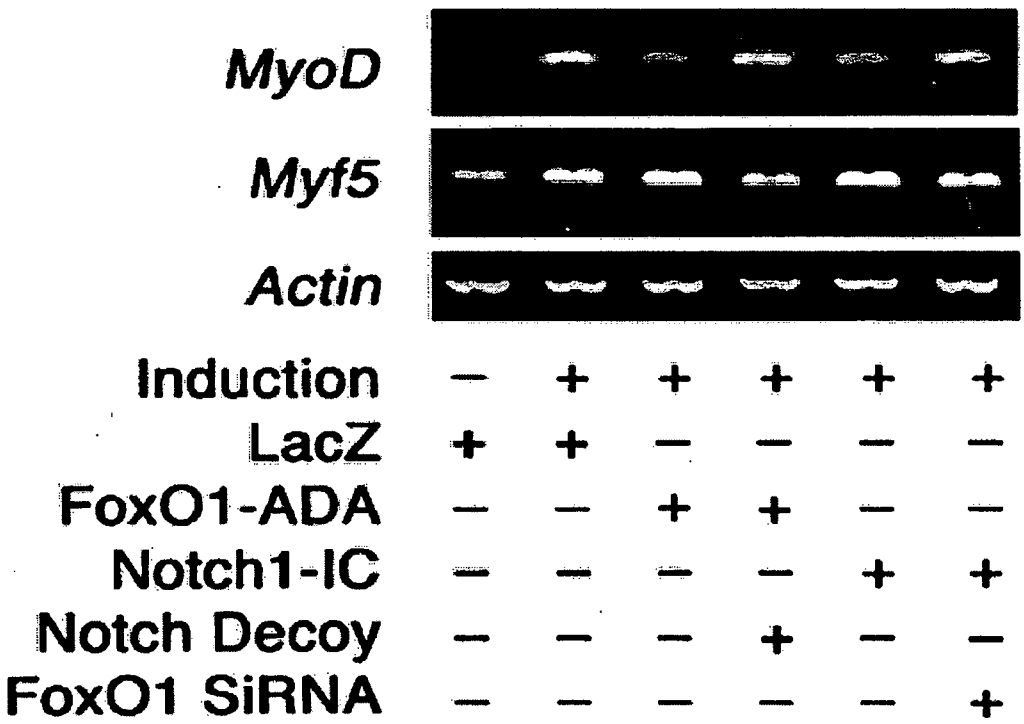
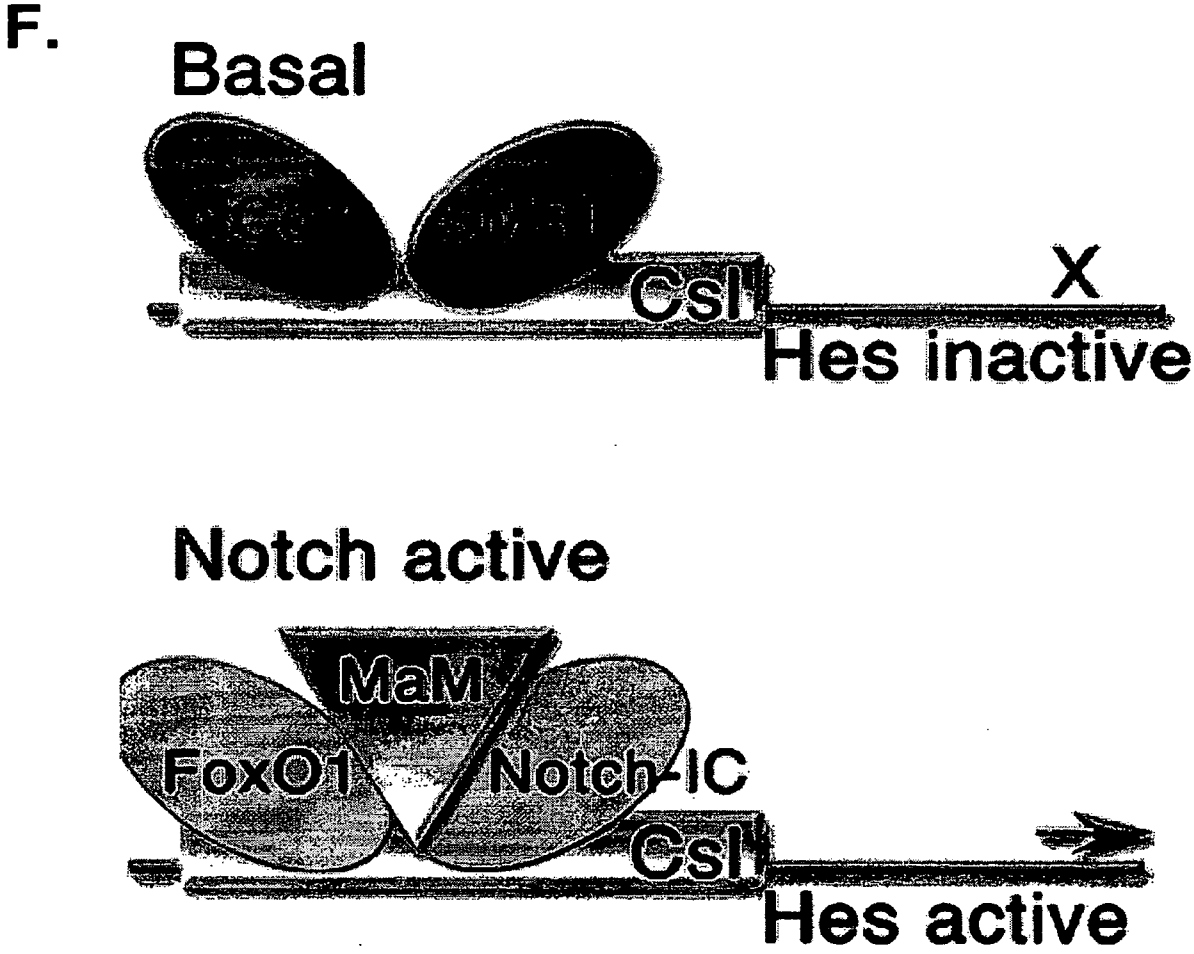

MyoD
Myf5
Actin
Induction − + + + + +
LacZ + + − − − −
FoxO1-ADA − − + + − −
Notch1-IC − − − − + +
Notch Decoy − − − + − −
FoxO1 SiRNA − − − − − +

FIG. 4D-E


F.
Basal
CsI
X
Hes inactive
Notch active
MaM
FoxO1
Notch-IC
CsI
Hes active

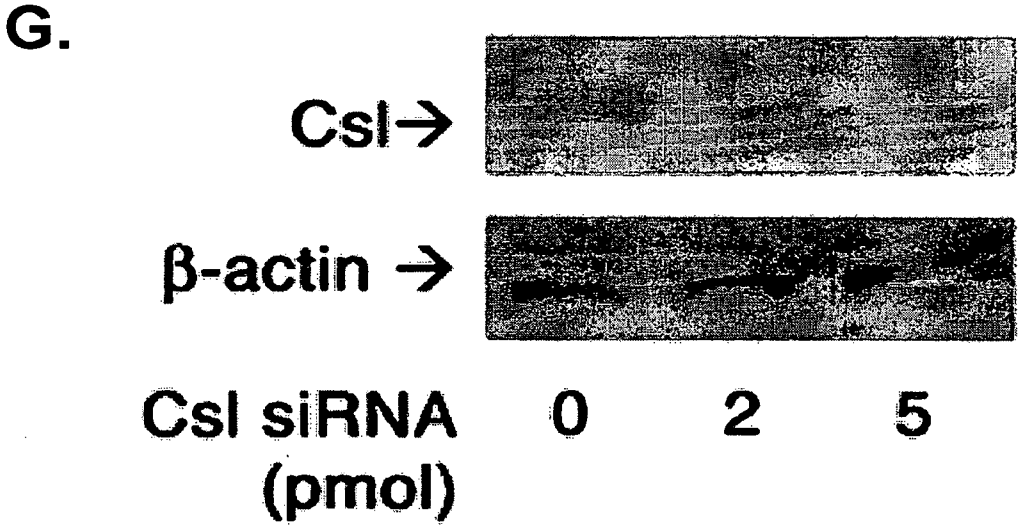

G.
CsI→
β-actin →
CsI siRNA (pmol)
0
2
5

FIG. 4F-G

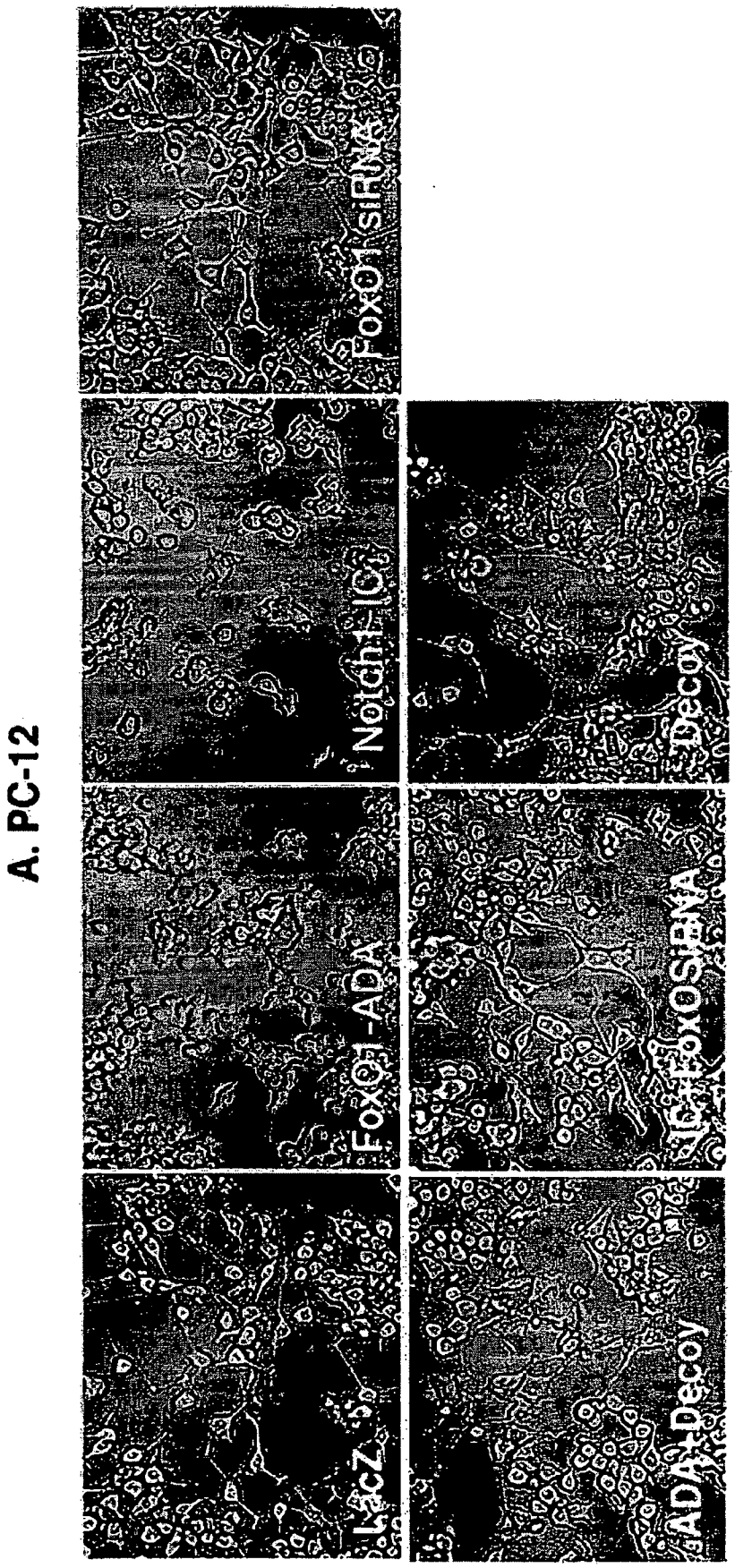

A. PC-12
LacZ
FoxO1-ADA
Notch1-IC
FoxO1siRNA
ADA Decoy
FoxO1siRNA
Notch1-IC Decoy

FIG. 5A

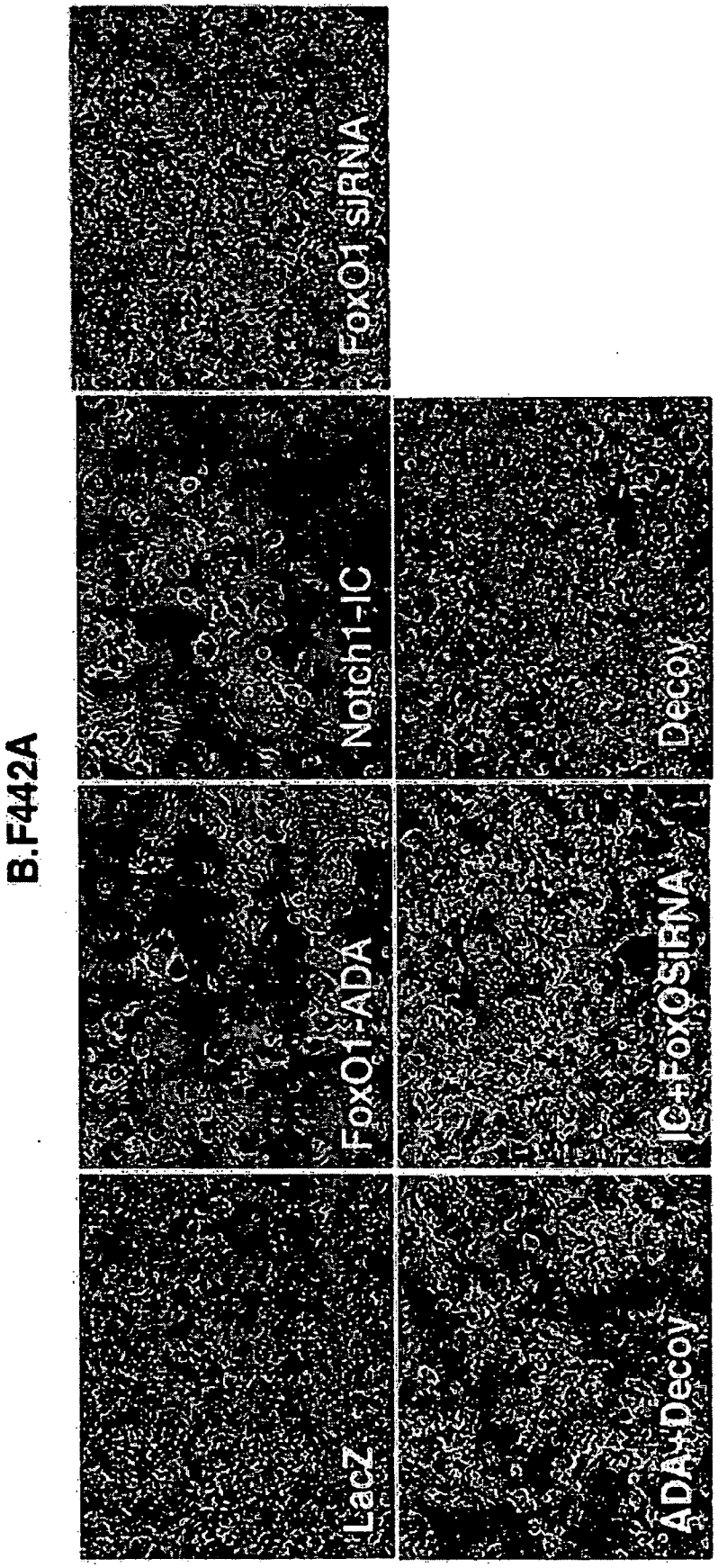

B.F442A
LacZ
FoxO1-ADA
Notch1-IC
FoxO1 siRNA
ADA+Decoy
Ie+FoxOSiRNA
Decoy

FIG. 5B

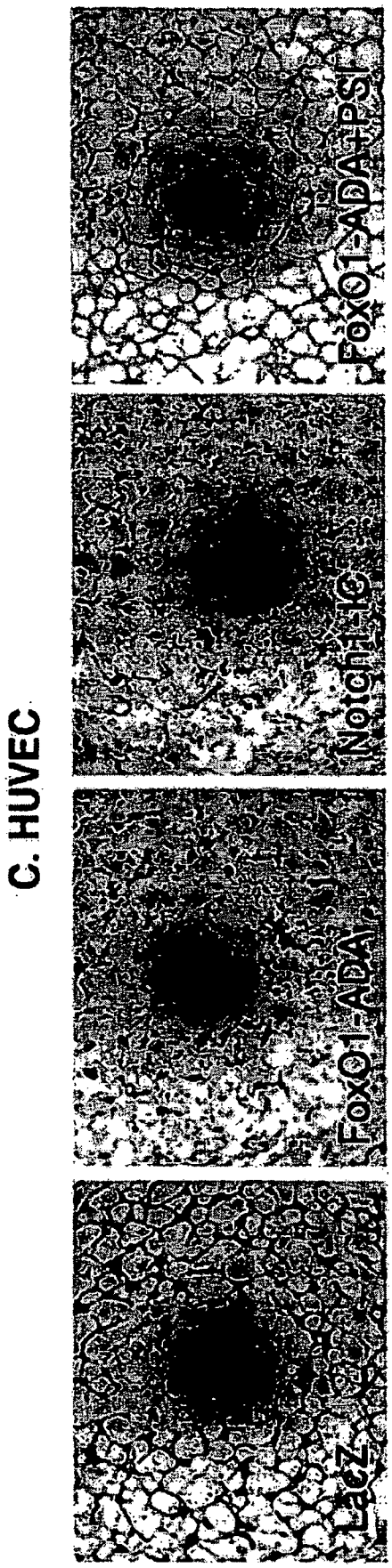


FIG. 5C

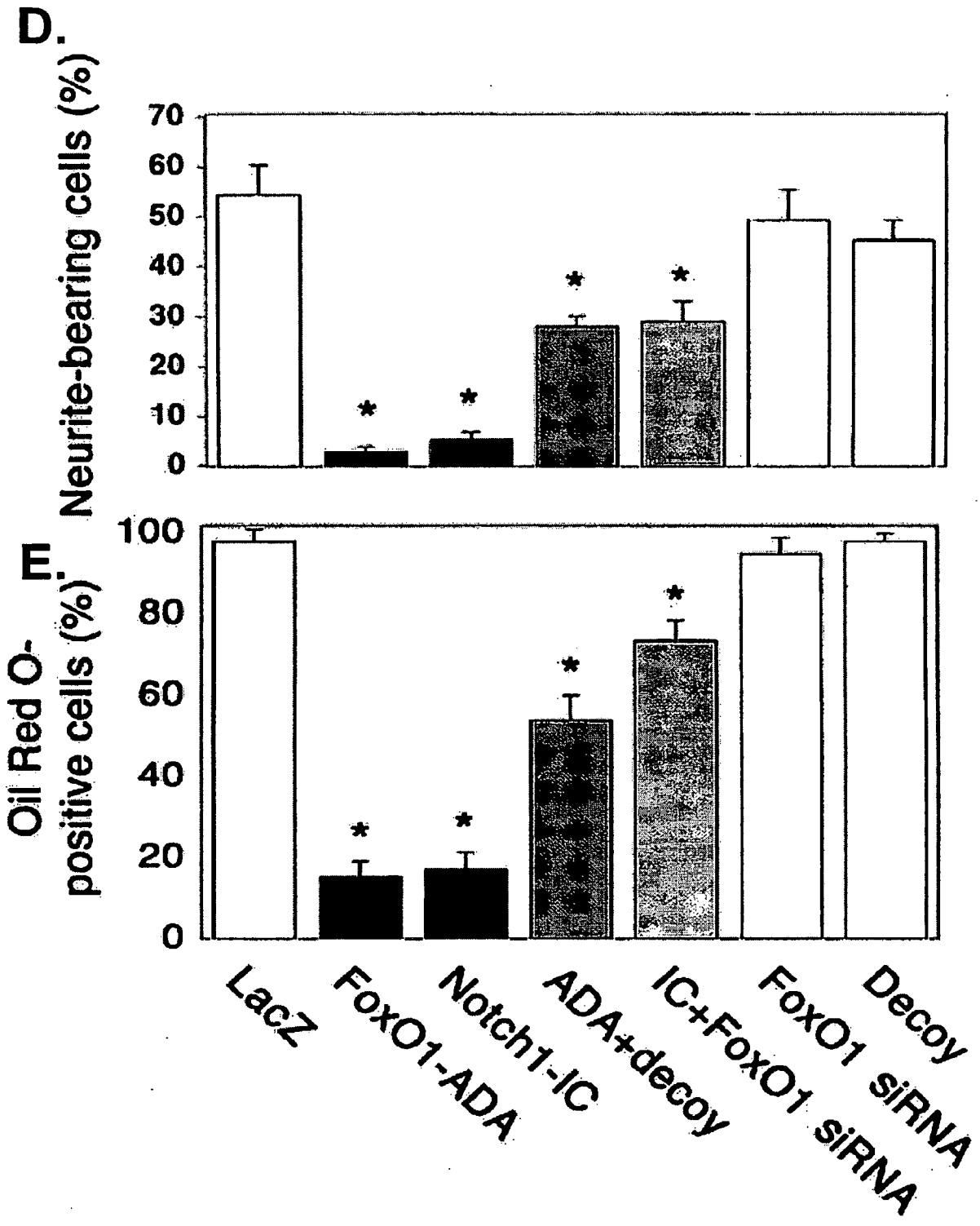

D.
Neurite-bearing cells (%)
70
60
50
40
30
20
10
0
E.
Oil Red O-positive cells (%)
100
80
60
40
20
0
*
LacZ
FoxO1-ADA
Notch1-IC
ADA+decoy
IC+FoxO1 siRNA
FoxO1 siRNA
Decoy

FIG. 5D-E

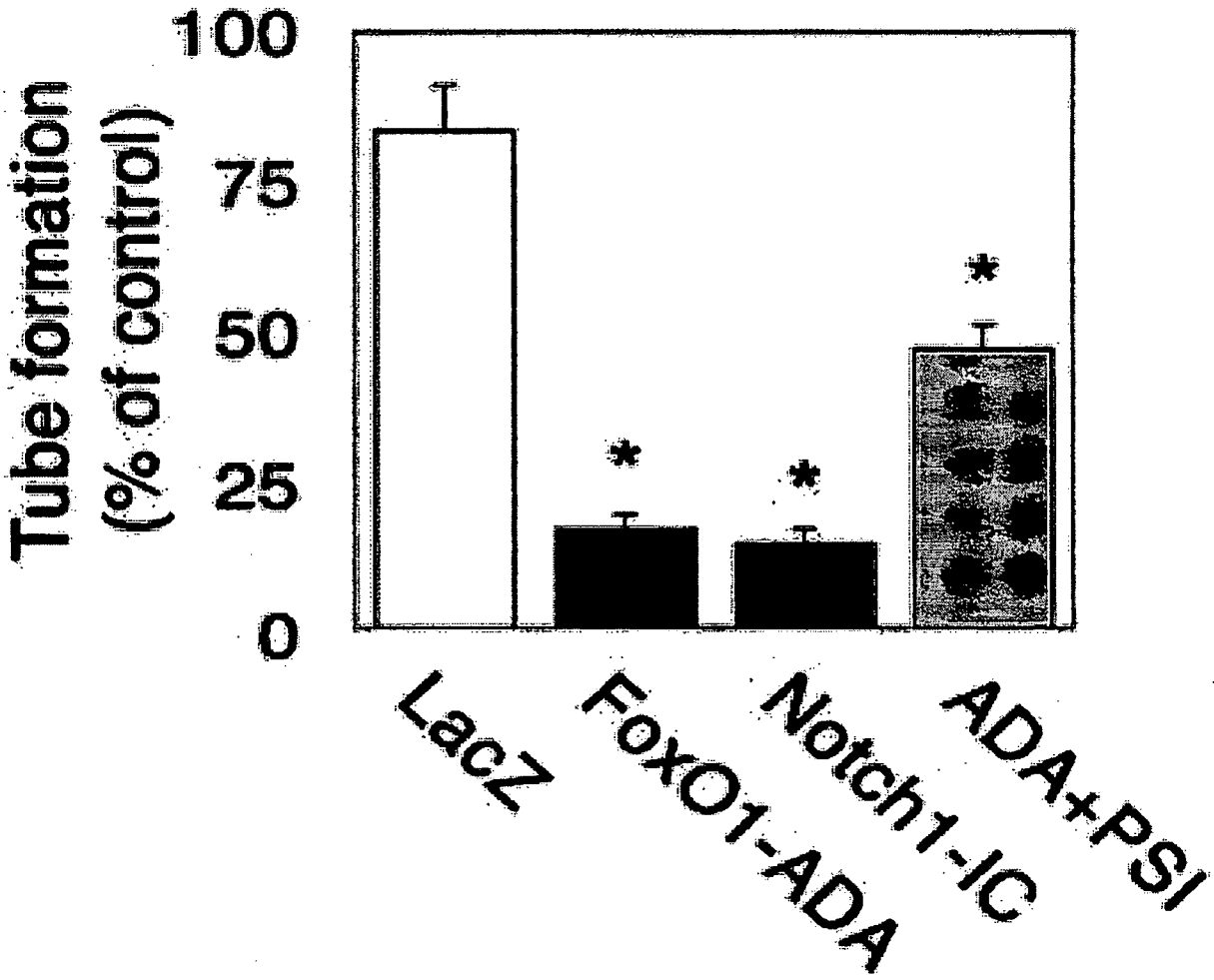

Tube formation
(% of control)
100
75
50
25
0
*
*
*
LacZ
FoxO1-ADA
Notch1-IC
ADA+PSI

FIG. 5F

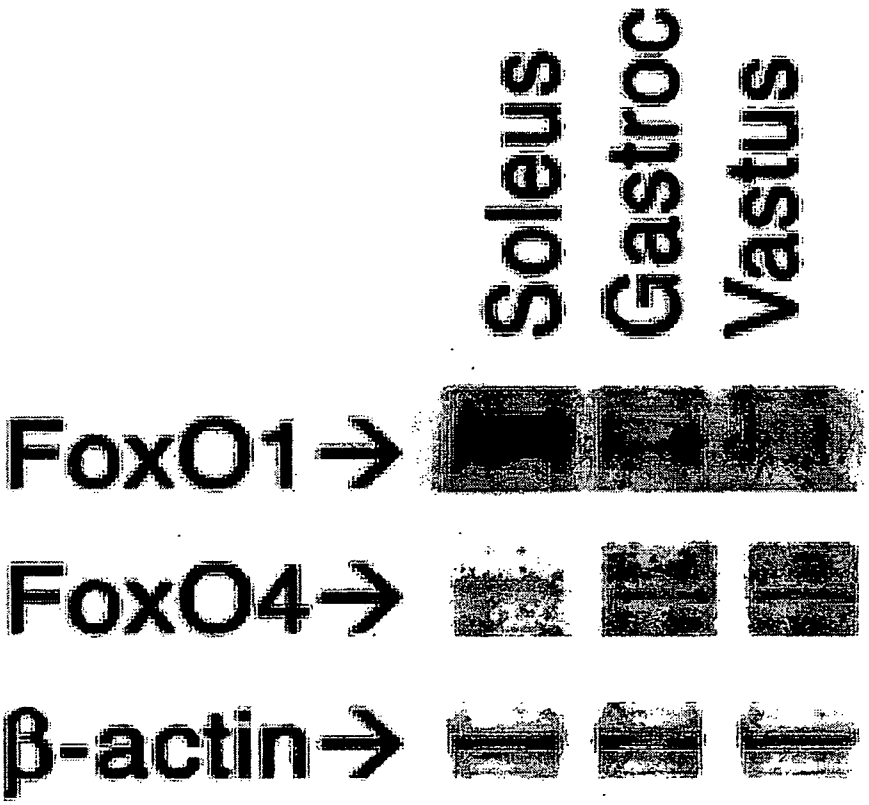


FIG. 6A

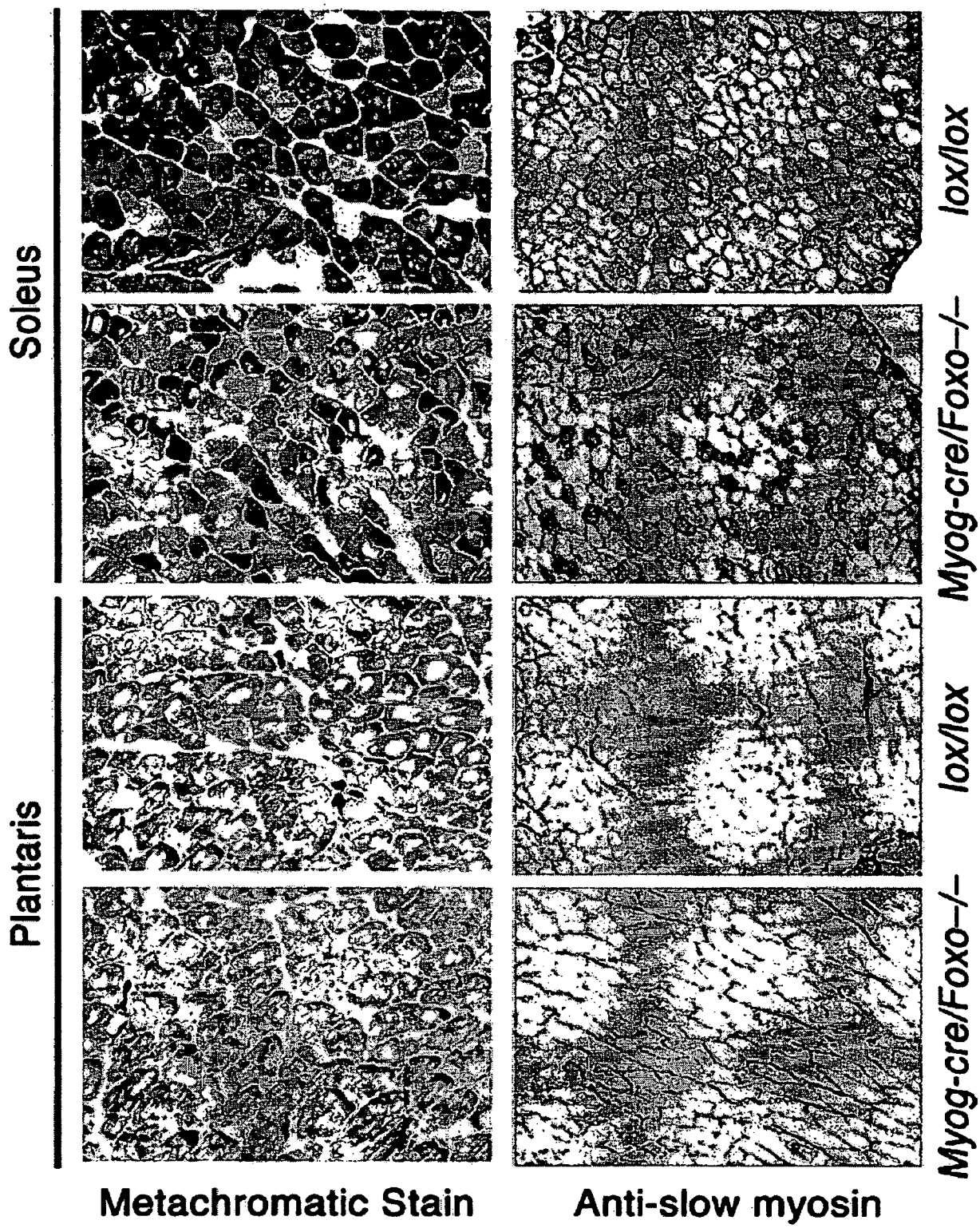

Soleus
Plantaris
lox/lox
Myog-cre/Foxo-/-
lox/lox
Myog-cre/Foxo-/-
Metachromatic Stain
Anti-slow myosin

FIG. 6B

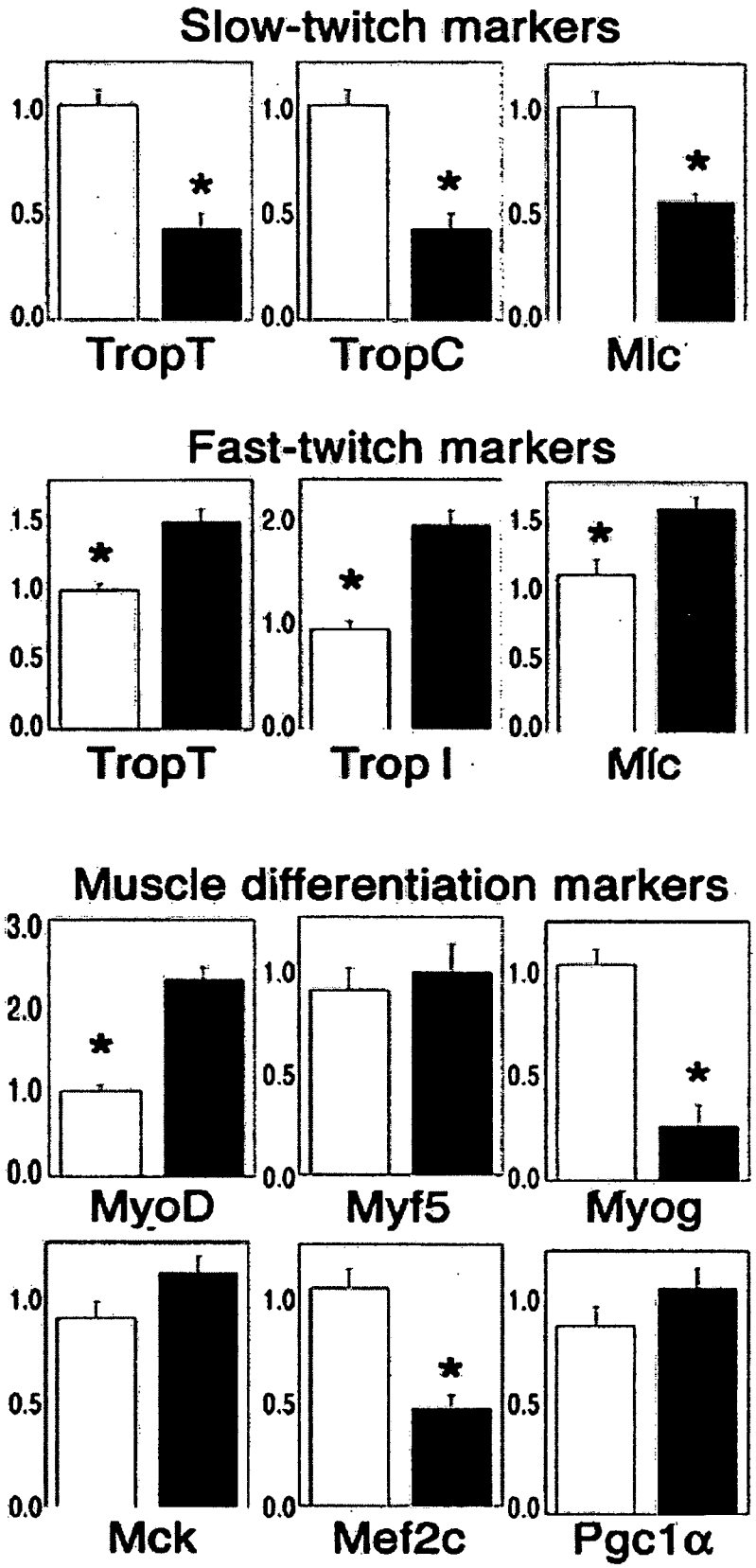

Slow-twitch markers
1.0
0.5
0.0
*
TropT
TropC
Mlc
Fast-twitch markers
1.5
2.0
TropT
Trop I
Mlc
Muscle differentiation markers
3.0
2.0
MyoD
Myf5
Myog
Mck
Mef2c
Pgc1α

FIG. 6C

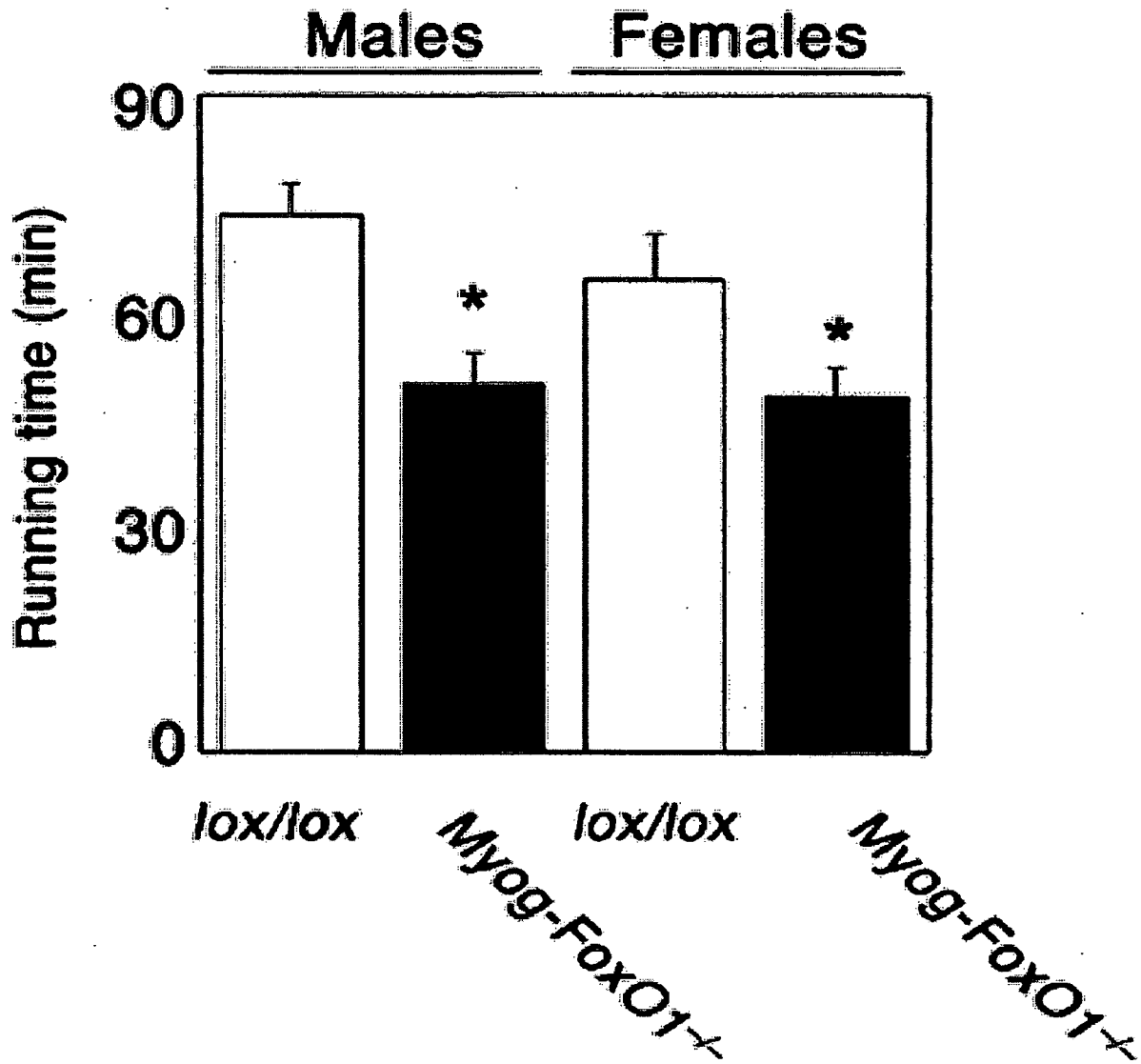

Males
Females
90
60
30
0
Running time (min)
*
*
lox/lox
Myog-FoxO1-/-
lox/lox
Myog-FoxO1-/-

FIG. 6D