

Adaptive changes in the transcription factor HoxA-11 are essential for the evolution of pregnancy in mammals

Vincent J. Lynch^{*†}, Andrea Tanzer^{**}, Yajun Wang[§], Frederick C. Leung[§], Birgit Gellersen[¶], Deena Emera^{*}, and Gunter P. Wagner^{*}

^{*}Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT 06511; [§]School of Biological Sciences, Kadoorie Biological Science Building, Department of Zoology, University of Hong Kong, Pokfulam Road, Hong Kong, China; [¶]Institute of Theoretical Chemistry, University of Vienna, Währingerstrasse, 1090 Vienna, Austria; and [†]Endokrinologikum Hamburg, Falkenried 88, 20251 Hamburg, Germany

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Evolutionary change in gene regulation can result from changes in *cis*-regulatory elements, leading to differences in the temporal and spatial expression of genes or in the coding region of transcription factors leading to novel functions or both. Although there is a growing body of evidence supporting the importance of *cis*-regulatory evolution, examples of protein-mediated evolution of novel developmental pathways have not been demonstrated. Here, we investigate the evolution of prolactin (PRL) expression in endometrial cells, which is essential for placentation/pregnancy in eutherian mammals and is a direct regulatory target of the transcription factor HoxA-11. Here, we show that (i) endometrial PRL expression is a derived feature of placental mammals, (ii) the PRL regulatory gene HoxA-11 experienced a period of strong positive selection in the stem-lineage of eutherian mammals, and (iii) only HoxA-11 proteins from placental mammals, including the reconstructed ancestral eutherian gene, are able to up-regulate PRL from the promoter used in endometrial cells. In contrast, HoxA-11 from the reconstructed therian ancestor, opossum, platypus, and chicken are unable to up-regulate PRL expression. These results demonstrate that the evolution of novel gene expression domains is not only mediated by the evolution of *cis*-regulatory elements but can also require evolutionary changes of transcription factor proteins themselves.

evolution of development | functional divergence | molecular evolution | *trans*-regulatory evolution

Developmental evolutionary studies have played an important role in elucidating the molecular mechanisms that give rise to phenotypic novelty and the evolution of gene regulation (1, 2). Mechanistically, embryonic development is governed by the precise temporal and spatial deployment of gene regulatory networks (1, 2). Thus, the evolution of development is fundamentally a question of the origin and evolution of gene regulation and regulatory networks. An emerging principle from evolutionary developmental studies is that adaptive mutations with phenotypic effects are much more likely to occur in *cis*-regulatory elements than in protein-coding genes (3–6). Indeed, there is ample evidence for the importance of *cis*-regulatory evolution (4–6), but it has also been shown that transcription factors do not remain functionally equivalent during evolution (7–9). This finding suggests that the evolution of transcription factors themselves may play an active role in the evolution of development and the origin of morphological innovations (10, 11). So far, however, no detailed evolutionary scenario has been elucidated that explains the role of transcription factor evolution. Here, we reconstruct some of the events that lead to the origin of pregnancy in eutherian mammals and demonstrate that adaptive changes in a transcription factor (HoxA-11) protein play an essential role.

One of the key evolutionary innovations of eutherian (placental) mammals is prolonged internal gestation and an invasive

embryo (pregnancy), adaptations made possible by the evolution of uterus and placenta. Knockout and knockdown studies have identified many genes important for pregnancy, including transcription factors, growth factors, and cell signaling molecules, such as prolactin (PRL) (12). In humans, PRL is one of the most dramatically induced genes in decidualized (differentiated) endometrial stromal cells, and decidual PRL is one of the most abundant secretory products in the amniotic fluid (13–17). Decidually expressed PRL has numerous functions, including regulation of uterine epithelial cell differentiation, trophoblast growth, angiogenesis, and modulating immune response (14, 16). A particularly important role for decidual PRL is the suppression of genes detrimental to pregnancy, such as interleukin 6 (IL-6) and 20 α -hydroxysteroid dehydrogenase (20 α -HSD), which promote inflammation and catabolize progesterone, respectively (13, 18, 19). Thus, the proper regulation of decidual PRL is essential for implantation and pregnancy.

Although the evolutionary mechanisms and timing of PRL recruitment into uterine expression are unclear, the molecular mechanisms leading to decidual PRL activation are well understood. Several genes have been identified that influence endometrial PRL expression, such as C/EBP β , FOXO1A, ETS1 and the progesterone receptor (20–24). The Abd-B-related Hox gene HoxA-11 is required to activate expression of PRL (V.J.L., B.G., and G.P.W., unpublished data). In addition to its role in PRL regulation, HoxA-11 is essential for the pre- and postnatal development of the female reproductive tract and is required for successful implantation of the mammalian blastocyst (25–28). Remarkably, a previous molecular evolutionary analysis of HoxA-11 suggested that an episode of positive Darwinian selection occurred in the stem-lineage of placental mammals, coincident with the origin of the uterus and pregnancy (29). This result suggests that adaptive evolution in HoxA-11 may have generated a novel PRL regulatory ability, possibly through the origin of new cofactor associations required for PRL expression in endometrium.

To clarify the evolutionary mechanisms that gave rise to uterine PRL expression, we have examined in detail the molecular and functional evolution of the PRL regulatory transcription factor HoxA-11. We show that endometrial PRL expression

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[†]To whom correspondence should be addressed at: OML 327, Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT 06511. E-mail: vincent.j.lynch@yale.edu.

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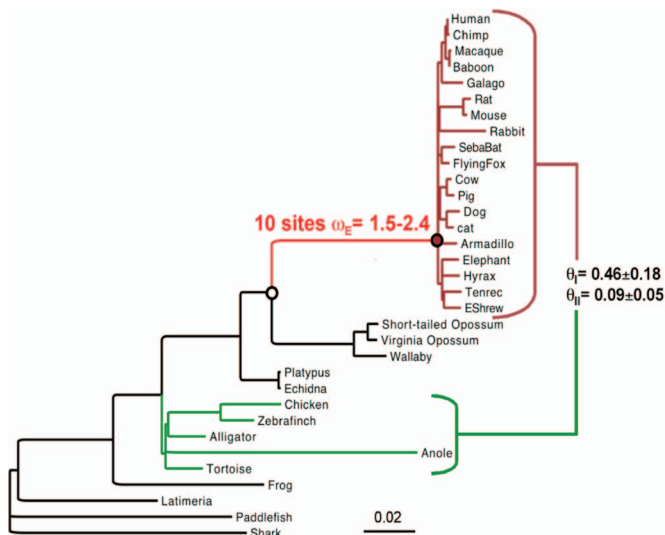


Fig. 2. Positive selection on HoxA-11 exon 1 in the stem-lineage of placental mammals. Eutherians are shown in dark red, with the stem-lineage of eutherians in red. Ten sites were identified under positive selection with $\omega_E = 1.5\text{--}2.4$ depending on how codon frequencies were modeled (equal or empirical). Type I (θ_I) and type II (θ_{II}) functional divergence was estimated between eutherians and sauropsids (shown in green). $\theta_I, P < 0.01$; $\theta_{II}, P < 0.05$. Branch lengths are proportional to the number of nonsynonymous substitutions per codon. The red filled and white filled circles indicate the location of the ancestral eutherian and therian sequences recreated for functional tests.

Together, these findings suggest that the HoxA-11 protein has acquired an additional functional constraint during the origin of placental mammals.

To test whether HoxA-11 genes in the Eutheria evolve under different selective constraints than in other vertebrate lineages, we estimated the coefficient of functional divergence (θ), which measures the difference in evolutionary rate at amino acid sites between two clades. Rejection of the null hypothesis ($\theta = 0$) is evidence for altered functional constraints between clades (40, 41). We found significant evidence of type I functional diver-

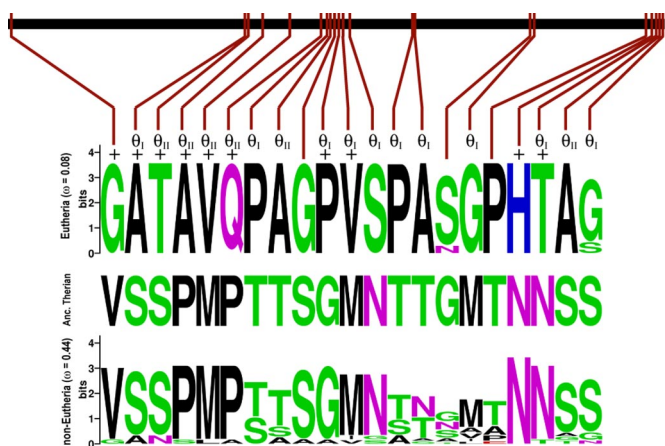


Fig. 3. Location of positively selected and functionally divergent sites in exon 1. The black bar represents exon 1 of HoxA-11, red marks indicate the relative locations of sites that were substituted in the stem-lineage of eutherians. The two sequence logos show site variation within eutherians (*Top*) and the noneutherians (*Bottom*). The inferred amino acids at these sites is shown for the reconstructed theroian ancestor (*Middle*). Amino acids are color coded by physicochemical property. +, site identified under positive selection; θ_I , type I site; θ_{II} , type II site. The rate of evolution of these sites is shown for eutherians and noneutherians.

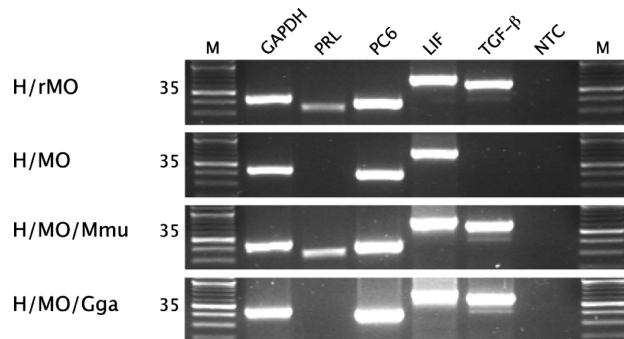


Fig. 4. Partial functional nonequivalence of human, mouse, and chicken HoxA-11 genes in endometrial cells. The expression of GAPDH (endogenous control) and marker genes of decidualized stromal cells (PRL; PC6, proprotein convertase 6; LIF, leukemia inhibitory factor; TGF- β ; NTC, no template control) were assayed by RT-PCR after hormone-induced differentiation and morpholino treatment. Treatments are shown on the left. H/rMO, hormone and random morpholino. H/MO, hormone and HoxA-11-specific morpholino. H/MO/Mmu, hormone and HoxA-11-specific morpholino rescued with mouse HoxA-11. H/MO/Gga, hormone and HoxA-11-specific morpholino rescued with chicken HoxA-11. M, 100-bp ladder. The number of PCR cycles used in the PCR is shown next to the treatments.

gence between eutherians and sauropsids ($\theta_I = 0.46 \pm 0.18, P < 0.01$; Fig. 1) and significant but weak evidence of type II functional divergence ($\theta_{II} = 0.09 \pm 0.05, P < 0.05$; Fig. 1). Type I sites (40) are amino acids that are conserved within the Eutheria but variable in the sauropsids, indicating they evolved new or stronger structural and functional constraints in Eutheria, whereas type II sites (41) are amino acids that are fixed in eutherians and sauropsids for different amino acids, indicating a functional switch. The greater degree of type I ($\theta_I = 0.46$) than type II ($\theta_{II} = 0.09$) functional divergence suggests that selection recruited weakly constrained amino acids into a novel function rather than amino acids that were under strong preexisting functional constraints. These data suggest that selection acted within existing structural and functional constraints on proteins to generate novel functions (Fig. 3).

The episode of adaptive evolution in HoxA-11 in the stem-lineage of eutherians and the absence of PRL expression in the uterus and oviduct of nonplacental mammals suggests that the ability to regulate PRL expression in the decidua might be a novel function of HoxA-11 in eutherians. We initially tested for functional differences between human and chicken HoxA-11 genes by using an *in vivo* knockdown/xenogene rescue system in cultured human endometrial stromal cells (hESCs). Briefly, hESCs were induced to decidualize with physiological levels of 17β -estradiol and progestin while intrinsic HoxA-11 expression was knocked down with a morpholino, followed by rescue with either mouse or chicken HoxA-11. Eight days after differentiation, genes known to be expressed in decidualized stroma, but not expressed in undifferentiated cells, were highly expressed in cells treated with both random-sequence control morpholino (Fig. 3) and untreated cells (data not shown). However, decidualized cells treated with the HoxA-11-specific morpholino failed to express PRL and TGF- β . To test whether HoxA-11 knockdown was reversible, we rescued the knockdown with mouse HoxA-11, which recovered both PRL and TGF- β expression (Fig. 3). We next rescued the knockdown with chicken HoxA-11, which does not have the derived amino acid replacements of placental mammals, and we recovered TGF- β expression but failed to rescue PRL expression (Fig. 4). These results suggest that the HoxA-11 protein underwent amino acid substitutions necessary for the derived ability to regulate PRL but that the

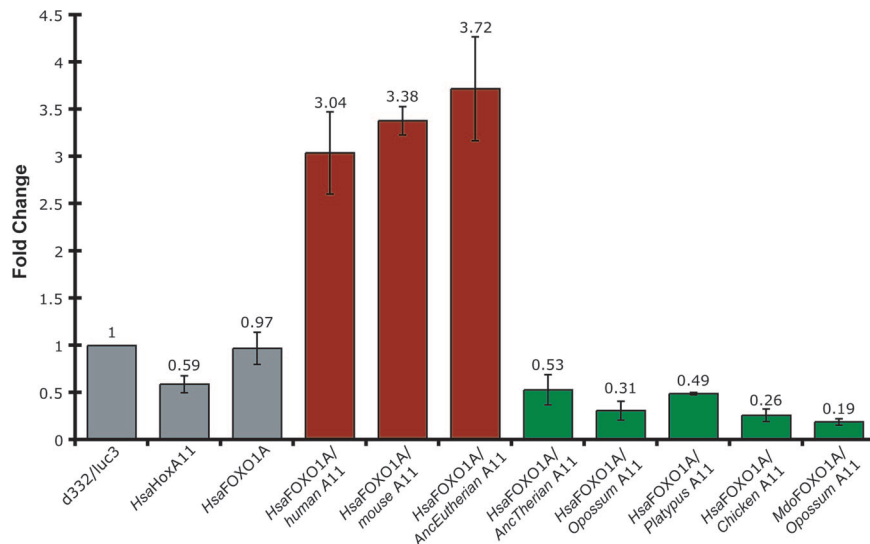


Fig. 5. Functional divergence in amniote *HoxA-11* genes ability to up-regulate expression from the decidual PRL enhancer. The ability of *HoxA-11* genes to activate expression from the decidual PRL enhancer was determined in a luciferase reporter construct using dPRL-332/luc3 (d332/luc3). Transfection with human *HoxA-11* alone (*HsaHoxA11*) repressed reporter gene expression. Transfection with human *FOXO1A* alone (*HsaFOXO1A*) had no effect on reporter gene expression. Cotransfection of human *FOXO1A* with human *HoxA-11* (human A11) or mouse *HoxA-11* (mouse A11) activated reporter gene expression. Cotransfection of human *FOXO1A* with opossum *HoxA-11* (opossum A11), platypus *HoxA-11* (platypus A11) or chicken *HoxA-11* (chicken A11) repressed reporter gene expression. Cotransfection of the reconstructed ancestral eutherian *HoxA-11* gene (*AncEutherian A11*) with the human *FOXO1A* gene activated reporter gene expression, conversely cotransfection of the reconstructed ancestral therian *HoxA-11* gene (*AncTherian A11*) with the human *FOXO1A* did not lead to activation of reporter gene expression. Expression levels are shown as fold changes relative to luciferase expression in cells transfected with the reporter gene (d332/luc3) and empty vector (pcDNA3.1). $n = 6$, mean \pm SEM.

ability to regulate TGF- β is ancestral for the amniote *HoxA-11* protein.

The knockdown/rescue approach described above is a powerful technique to test for functional divergence, particularly in nonmodel organisms, but it can be complicated by complex auto- and cross-regulatory interactions that can interfere with the knockdown, the rescue, or both. Therefore, it is desirable to test functional divergence in more controlled assays, particularly outside of the cell type of interest, once functional differences are identified. To this end, we used a luciferase reporter assay in HeLa cells to test specifically the ability of *HoxA-11* genes from the major amniote lineages to activate transcription from the decidual PRL enhancer. Transfection with *HoxA-11* alone repressed reporter gene expression, consistent with previous studies that found that the *HoxA-11* protein acts as an intrinsic repressor (42). Cotransfection of mouse or human *HoxA-11* with *FOXO1A* led to dramatic activation from the PRL enhancer (Fig. 5), consistent with the rescue results in hESCs. Next, we tested whether the opossum, platypus, and chicken *HoxA-11*, which lack the derived placental-specific adaptive amino acid substitutions, activated luciferase expression from the decidual PRL enhancer. Remarkably, unlike mouse and human *HoxA-11*, the orthologs from opossum, platypus, and chicken, species that lack PRL expression in uterus and oviducts, repressed luciferase expression when cotransfected with *FOXO1A* (Fig. 4). Finally, we tested whether opossum *HoxA-11* could activate expression from the dPRL enhancer when paired with the opossum *FOXO1A* gene. Similarly to the opossum *HoxA-11* paired with human *FOXO1A*, opossum *HoxA-11*/opossum *FOXO1A* failed to activate luciferase expression (Fig. 5).

To test specifically whether the amino acids identified from the statistical analysis as under positive selection and/or functional divergence were sufficient to confer PRL regulatory ability on a *HoxA-11* gene that lacks PRL regulatory functions, we reconstructed the ancestral therian (i.e., the protein from the last common ancestor of humans and opossum) *HoxA-11* gene by maximum likelihood and tested its ability to activate luciferase

expression from the decidual PRL enhancer. As expected, the ancestral therian *HoxA-11* gene, which lacks the selected and functionally divergent amino acid sites, was unable to cooperate functionally with *FOXO1A* to up-regulate PRL expression (Fig. 5). However, the ancestral eutherian *HoxA-11* gene, which has the selected and functionally divergent amino acid sites and was reconstructed without ambiguity (Bayesian posterior probability of 1.0), did have PRL activation ability (Fig. 5). These results show that at least some of the amino acid substitutions in the stem-lineage of placental mammals generated a novel functional interaction with *FOXO1A* that was used to recruit PRL expression into the uterus of placental mammals.

Although protein-mediated evolution of developmental pathways is rarely excluded as a means of gene regulatory evolution, the contribution of protein change to the origin of novel gene regulatory networks is generally not considered to play a major role in evolution. The primary argument against protein-mediated evolution of gene regulatory networks is the negative pleiotropic effects of mutations that are ascribed to changes in protein-coding genes (3, 4). For example, given the multiple functions of *HoxA-11* in blood cell differentiation (43) and development of the body axis (44), limbs (25, 45), kidney (46, 47), and male (27, 28) and female reproductive systems (25, 27, 28), it seems unlikely that a novel function could emerge in endometrial cells without simultaneously having deleterious effects in these other contexts. However, our data indicate that selection acted to maintain ancestral functions during the emergence of a novel function by recruiting amino acid sites that were previously under weak functional constraints and thus free to acquire novel functions. The identification of an episode of adaptive evolution in *HoxA-11* coincident with the origin of a novel function demonstrates a clear link between adaptive protein evolution and the emergence of a novel function. This and other examples of functional divergence among transcription factors (7–9) indicate that the evolution of proteins themselves actively contributes to the evolution of development.

Materials and Methods

Gene Expression Surveys in Elephant, Opossum, and Chicken. Expression of PRL, PRLR, FOXO1A, IL-6, and 20 α -HSD in the pregnant (stage 32) opossum (*M. domestica*) endometrium, myometrium, placenta, ovary, and fetus was assayed by RT-PCR. Similarly, the expression of PRL in sexually mature (egg-laying) female chickens (*Gallus gallus*) and in the placental tissue of an African elephant (*L. africana*) was assayed by RT-PCR. Details are provided in the *SI Text*.

Molecular Evolutionary Analysis of HoxA-11. HoxA-11 genes were identified from BLAST searches of whole-genome databases at National Center for Biotechnology Information, BLAT University of California at Santa Cruz, and BLAST ENSEMBL; see *SI Text* for a list of included species. We used codon-based maximum-likelihood models of coding sequence evolution implemented in CODEML in the PAML 4 package of programs (49) to test for lineages and amino acid sites under positive selection. Sites were classified as being under positive selection if they were identified from the Bayes Empirical Bayes (BEB) method with a posterior probability of >0.90 under model A. Initial branch lengths for the input trees were determined under the one-ratio model (M0) and used in further analyses. To ensure convergence of the maximum-likelihood estimates generated under the branch-specific and branch-site models we altered the starting values of the d_N/d_S ratio, the transition:transversion ratio, and the equilibrium codon frequencies ($f = , f_{61}$) and reanalyzed the data. Difference in selective pressure between putatively positively selected sites in extant mammals and other species was obtained by averaging the sitewise d_N/d_S values from those sites with the site-specific model M3 with three rate categories. Ancestral sequences were inferred with CODEML, which uses maximum likelihood and an empirical Bayes approach to estimate ancestral character states, and the phylogeny shown in Fig. 2. The Bayesian posterior probability of the reconstructed therian ancestral sequence (AncTherian) was 0.96, and the probability of the eutherian (AncEutherian) ancestral sequence was 1.0. Functional divergence was tested with DIVERGE 2 (50). A limitation of functional divergence analyses is the requirement of at least four taxa per clade for estimation of the coefficient of functional divergence (θ). Given this limitation, we only tested for functional divergence between placental mammals and birds/reptiles.

HoxA-11 Expression Vector Construction. We amplified axon 1 of *HoxA-11* by PCR from the genomic DNA of human (*Homo sapiens*), mouse (*Mus musculus*), opossum (*Didelphis virginiana*), platypus (*Ornithorhynchus anatinus*), and chicken (*G. gallus*) by using previously described degenerate primers (29). Exon 2 is identical in amino acid sequence for these species, therefore we fused the human exon 2 sequence to exon 1 of the above species by using primer overlap PCR mutagenesis; C-terminal His₆ tags were added by PCR mutagenesis. His-tagged PCR products were cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen) and verified by sequencing. Proper expression of tagged HoxA-11 genes was checked in HeLa cells with immunocytochemistry using an anti-His antibody. The ancestral therian (AncTherian) and

ancestral eutherian (AncEutherian) genes were synthesized by GeneScript Corp. with human optimized codon usage and ligated into pcDNA3.1.

In Vivo HoxA-11 Knockdown/Xenogene Rescue. Tests of functional nonequivalence were performed in a telomerase-immortalized human endometrial stromal cell line (CRL-4003; American Type Culture Collection) treated either with an antisense morpholino (GeneTools) generated against the human HoxA-11 5'-UTR or a random sequence morpholino. Morpholinos were delivered by using EndoPorter (GeneTools) according to the manufacturer's protocols. Twelve hours after morpholino treatment, cells were differentiated with 10⁻⁹ M 17 β -estradiol and 10⁻⁸ M medroxyprogesterone acetate, and total RNA was collected after 8 days. HoxA-11 knockdown in endometrial cells was rescued with either a mouse HoxA-11 or chicken HoxA-11 expression vector. Efficacy of morpholino knockdown and rescue was determined by assaying the expression of genes known to be induced during differentiation, including PRL, leukemia inhibitory factor, proprotein convertase 6, and TGF- β , and the expression of the housekeeping gene GAPDH by RT-PCR after RNA extraction (RNeasy; Qiagen) and cDNA preparation (High-Capacity Reverse Transcriptase kit, Applied Biosystems). RT-PCR primers were designed to span at least one intron.

Transient Transfection and Luciferase Reporter System. HeLa cells were grown in 24-well culture plates in DMEM supplemented with 10% FBS. At 80% confluence, cells were transiently transfected with *trans-IT-LT1* (Mirus) according to the manufacturer's instructions by using 100 ng of the dPRL luciferase reporter construct dPRL-332/Luc3, which contains the wild-type dPRL promoter sequence -332 to +65 relative to the human dPRL transcriptional start site (51). Depending on treatment, cells were cotransfected with either 200 ng of empty pcDNA3.1(+) (vehicle control) or 100 ng of the FOXO1A expression vector pcDNA3.1(+)-FOXO1A (Addgene plasmid 13507) and 100 ng of one of the HoxA-11 expression vectors described above. In addition, the effect of both FOXO1A alone and HoxA-11 alone on reporter gene expression was assayed by transfection with the reporter plasmid and 100 ng of pcDNA3.1(+)-FOXO1A/100 ng of empty pcDNA3.1(+), or 100 ng of pcDNA3.1(+)-hHoxA-11/100 ng of empty vector. Forty-eight hours after transfections, total mRNA was harvested, and cDNA was prepared by using a High-Capacity Reverse Transcriptase kit (Applied Biosystems). Luciferase expression was assayed with quantitative real-time PCR (qRT-PCR) by using a luciferase-specific and GAPDH *TaqMan* primer/probe pair (Applied Biosystems). Each experiment was repeated twice, with three replicates per experiment. qRT-PCRs were also performed in triplicate.

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