

IDENTIFICATION OF MOLECULAR SIGNALING PATHWAYS UNDERLYING
HUMAN ENDOMETRIAL STROMAL CELL DIFFERENTIATION

BY

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DISSERTATION

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ABSTRACT

Decidualization, the differentiation of endometrial stromal cells, is essential for a successful pregnancy. Although the steroid hormones estrogen (E) and progesterone (P) are known to control decidualization, the precise mechanisms via which these hormones act to control this differentiation process are poorly understood. We used primary cultures of human endometrial stromal cells (HESC) to analyze the role of estrogen receptor alpha (ESR1) and progesterone receptor isoforms (PRA and PRB) in human decidualization.

Previous studies established that HESC, when treated with the differentiation cocktail containing E, P, and a cyclic adenosine monophosphate (cAMP) analog, cease proliferation and undergo differentiation. In the present study, when ESR1 expression was silenced, the HESC continued to proliferate in the presence of the differentiation cocktail and their differentiation was severely inhibited. Gene expression profiling revealed that, in the absence of ESR1, the expression levels of several cell cycle regulatory factors were increased and those of specific cell cycle inhibitors were decreased. Our study also revealed that ESR1 promoted the expression of key regulators of HESC differentiation (*PGR*, *FOXO1* and *WNT4*). Expression of these targets was dependent on the addition of cAMP, suggested a functional link between cAMP and ESR1 signaling. Using a proteomic approach, we identified MED1 as a target of cAMP-activated protein kinase (PKA) during HESC differentiation. The PKA-dependent phosphorylation of MED1 enhanced its ability to interact efficiently with ESR1. Furthermore, loss of MED1 expression inhibited HESC differentiation with parallel impairment in the expression of a subset of ESR1 target genes. Addition of cAMP increased recruitment of MED1 to the ESR1 binding regions of a target gene (*WNT4*). Collectively, these results indicated that, during decidualization, ESR1 suppresses HESC proliferation and promotes their differentiation via interactions with MED1, which is activated in response to cAMP signaling.

Progesterone, acting through its receptors, is essential for the precise regulation of the endometrial processes required for a successful pregnancy, including decidualization. However, the specific roles of the progesterone receptor isoforms, PRA and PRB, during endometrial differentiation in the human have remained unknown. A major focus of my project was to shed light on the roles of the receptor isoforms by identifying their cistomes and correlated gene expression profiles during differentiation of human stromal cells. We expressed PRA and PRB

individually after silencing endogenous progesterone receptors so that the roles of the isoforms could be analyzed independently as well as jointly. Identification of the cistromes of PRA and PRB using chromatin immunoprecipitation (ChIP) followed by high throughput sequencing (ChIP-seq), revealed that PRB cistrome was larger, covering that of PRA, at an early time point of *in vitro* differentiation of human endometrial stromal cells. Our *de novo* motif analysis showed that, both isoforms bind to the same DNA sequence motif, progesterone response element (PRE). In addition to the PRE, they had both common and distinct nearby motifs where other transcription factors might bind. Many molecules previously identified as progesterone target genes and known to regulate stromal differentiation were found to contain PRA and PRB binding sites, including *BMP2*, *HAND2* and *HOXA10*, confirming the validity of our method.

Our comprehensive gene expression analysis suggested that PRA and PRB regulated overlapping and distinct sets of genes. When PRA and PRB were expressed together, PRB was the prevalent isoform, while both isoforms influenced each other's transcriptional activity. Our findings suggested that PRB was a more effective transcription factor than PRA in human stromal cell differentiation by the extent to which it interacted with the genomic binding sites and regulated downstream gene networks. Collectively, our first line of analysis provided an insight into the early molecular mechanisms regulated by progesterone receptor isoforms during stromal differentiation. With this study, we confirmed some of the known roles of progesterone, such as regulation of angiogenesis, proliferation and apoptosis. We could also distinguish signaling networks downstream of each isoform and identify new molecules, such as IRS2, as possible direct targets of progesterone signaling for the establishment of a successful pregnancy.

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TABLE OF CONTENTS

CHAPTER I: Introduction	1
Uterine functions during early pregnancy.....	1
Estrogen and progesterone receptors and their known functions during decidualization	2
An <i>in vitro</i> system to study differentiation of human endometrial stromal cells.....	3
Roles of E and P and their receptors during HESC decidualization.....	5
Role of cAMP-dependent signaling during decidualization.....	6
References.....	8
CHAPTER II: Role of ESR1 and cAMP during human endometrial stromal cell differentiation	14
Abstract.....	14
Introduction.....	14
Materials and Methods.....	16
Results.....	20
Discussion	27
References.....	31
Figures and tables	37
CHAPTER III: Role of progesterone receptor isoforms during human endometrial stromal cell differentiation.....	52
Abstract.....	52
Introduction.....	52
Materials and Methods.....	54
Results.....	57
Discussion	63
References.....	69
Figures and tables	75
CHAPTER IV: Conclusions and future perspectives	101
ESR1 plays a critical role in human decidualization	101
Role of PR isoforms in human decidualization	102
Identification of potential direct targets of PRA and PRB during human decidualization.....	103
P regulation of energy metabolism during decidualization	104
References.....	107

CHAPTER I

INTRODUCTION

Uterine functions during early pregnancy

Endometrium is a unique adult tissue because of its capacity to proliferate and differentiate in a cyclical fashion during each menstrual cycle. The cycle of proliferation, differentiation, regression and regeneration is repeated hundreds of times during the reproductive years of a female [1-3]. This unique feature of endometrium is actually a preparation for a possible pregnancy which is synchronized with the cell division cycles of a fertilized egg and its migration from the fallopian tubes to the lumen of the uterus so that the endometrium is ready or “receptive” when the blastocyst arrives for implantation (Fig. 1) [4, 5]. Implantation is an essential step for a successful pregnancy [4]. It proceeds through distinct phases of maternal-embryo interactions, involving apposition, attachment and invasion stages [5]. The attachment of the embryo to the uterine epithelium is accompanied by a dramatic transformation of the underlying stromal compartment [5]. During this process, known as decidualization, fibroblastic, non-secretory stromal cells undergo differentiation into morphologically and functionally distinct decidual cells. [3-5]. The decidual tissue supports and nourishes the embryo until placenta formation. Additionally, it modulates local immune response to protect the embryo and regulates trophoblast invasion [5-7].

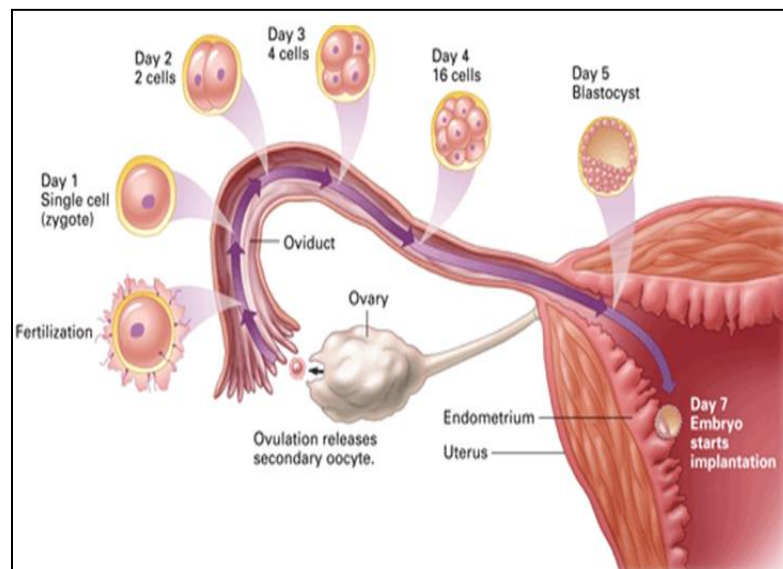


Figure 1.1: Diagram of the uterus and the journey of the blastocyst.
Adapted from <http://proctornet.com/text/chapter33/33images/33-09.gif>

In the human, induction of decidualization does not require the presence of the blastocyst; it commences in the secretory phase of every menstrual cycle and continues if pregnancy occurs. The menstrual cycle is regulated by the steroid hormones estrogen (E) and progesterone (P). The human endometrium first undergoes an E-dominated proliferative phase followed by a P-dominated secretory phase [8] during which decidualization of endometrial stromal cells, termed predecidualization, occurs independent of conception. If there is no conception, the circulating P levels go down in the late secretory phase, leading to the shedding of the decidualized tissue layer, triggering menstruation. If pregnancy occurs, decidualization progresses further and spreads through the endometrium, helping trophoblast invasion and placenta formation [9].

Impaired decidualization is associated with endometrial and reproductive diseases and dysfunctions, such as menstrual disorders, endometriosis, infertility and early pregnancy loss [7, 10]. Therefore, a better understanding of the decidualization process will give valuable information to address the underlying deficiencies in some of these endometrial disorders.

Estrogen and progesterone receptors and their known functions during decidualization

E and P act, at least in part, through their nuclear receptors. These receptors belong to the family of ligand activated transcription factors [11]. Hormone binding causes a conformational change in the receptor, which triggers dimerization and DNA binding of the receptor accompanied by recruitment of co-regulators and associated chromosome remodeling complexes, leading to modulation of gene expression [11, 12]. Two receptor subtypes have been identified for estrogen: estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2) [13]. For progesterone, there are two receptor isoforms: progesterone receptor A (PRA) and progesterone receptor B (PRB) [14]. Various gene knockout mouse models have provided valuable information to understand the roles of these steroid receptors and their downstream targets in decidualization. For example, female mice lacking progesterone receptor A or its downstream target *Hoxa10* are both defective in decidualization and are infertile [15-17]. On the other hand, the progesterone receptor B null mouse is fertile with normal decidualization response [18]. Estrogen receptor alpha knock out female mouse is infertile and defective in

decidualization (unpublished data). Estrogen receptor beta null mouse, which is subfertile, is reported to retain normal uterine functions including decidualization and can support normal pregnancy [19]. The steroid receptor coregulators play critical roles in regulating stromal differentiation as well. For example, the steroid receptor co-activator-1 null and Repressor of Estrogen Activity (REA) homozygous mutant females exhibit defects in decidualization [20, 21]. These *in vivo* data support the importance of steroid hormone receptors, PRA and ESR1 in particular, in regulation of decidualization process. However, the molecular mechanisms downstream of these receptors are still not well understood.

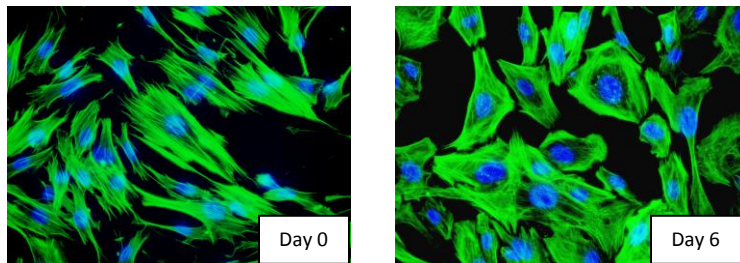
An *in vitro* system to study differentiation of human endometrial stromal cells

In order to study the molecular pathways underlying decidualization in human, an *in vitro* system using primary cultures of proliferative phase human endometrial stromal cells (HESCs) are used. These cells are isolated from endometrial biopsies of 18-35 years old women volunteers. Stromal cells are isolated by enzymatic digestion and placed in culture media containing serum. It is shown that these primary cultures retain their biological characteristics for at least ten passages and exhibit a decidualization phenotype that is very similar to endometrial stroma *in vivo* at both biochemical and morphological levels [22, 23]. Addition of a differentiation cocktail composed of E, P, and a cyclic AMP analog 8-Br-cAMP triggers differentiation of HESCs [24-26]. The fibroblastic stromal cells undergo a distinct morphological change as they are transformed into larger, and round decidual cells (Figure 2a). This transformation in the cellular architecture is accompanied by a dramatic rise in the expression of known biomarkers of decidualization (Figure 2b). A number of biochemical markers, such as prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP1), have been widely used to monitor the progress of the decidualization program *in vitro* [22, 27].

Use of this *in vitro* system to study human decidualization has many advantages. It is possible to manipulate the treatment conditions, such as addition or removal of steroid hormones, morphogens, second messengers or other regulatory molecules. SiRNA-mediated strategies can be used easily to alter specific gene expression to conduct functional analysis. This *in vitro* system has some limitations too. It lacks the signaling events contributed by the epithelium and

the implanting blastocyst. Overall, it is a very useful system to study human stromal cell differentiation.

A.



B.

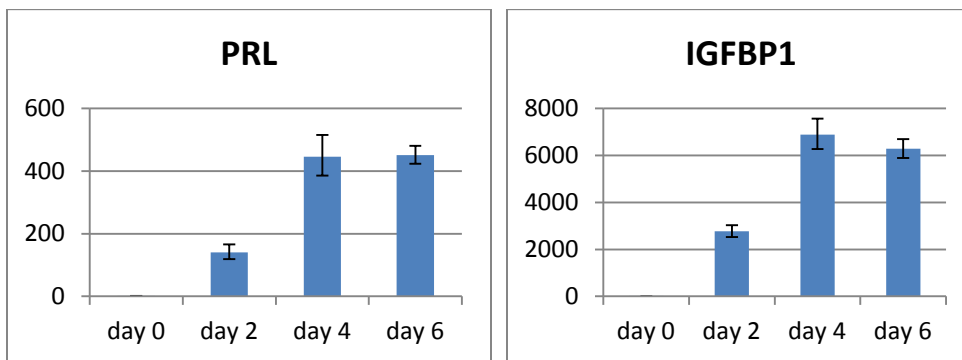


Figure 1.2: *In vitro* differentiation of HESC

HESC were grown in 2% charcoal-stripped serum for 2 days. Cells were then treated with the differentiation cocktail containing 0.5mM 8-bromo-cAMP, 1uM P and 10nM E for 0-6 days.

A. HESCs were seeded on 4-well chamber slides and subjected to *in vitro* differentiation for 6 days. DAPI (blue) and Phalloidin (green) staining of HESCs are shown. Day 0 and day 6 designates the time point prior to and 6 days following differentiation cocktail addition, respectively.

B. HESCs were harvested at different time points following differentiation cocktail treatment. Total RNA was isolated and cDNAs were prepared. cDNAs were subjected to real-time PCR using gene specific primers for PRL and IGFBP1. Day 0 designates the time point prior to differentiation cocktail addition. PCR data are normalized to 36B4 mRNA levels. Relative fold changes were normalized with respect to Day 0.

Roles of E and P and their receptors during HESC decidualization

The ovarian steroids E and P are the main regulators of the decidualization process. These hormones act through their nuclear receptors. Estrogen receptor subtypes, ESR1 and ESR2 are expressed in the uterine stroma. ESR1 is the dominant subtype. Its expression is high during proliferative phase and it continues during early secretory phase. By late secretory phase, the ESR1 expression is very much reduced. The ESR2 expression, on the other hand, is relatively low at all stages of the menstrual cycle. Its highest expression is observed in late secretory phase [28, 29]. Development of global *Esr1*-null mice in the Chambon laboratory and conditional *Esr1* null mice in our laboratory revealed that decidualization fails to occur in the absence of ESR1 (unpublished data). A recent study using human endometrial stromal cells showed that knockdown of the estrogen receptor coregulator REA, repressor of estrogen receptor activity, enhanced the differentiation of HESC, as shown by the increased expressions of biomarkers of decidualization [30]. Although these findings suggest that ESR1 plays an essential role during decidualization; its role in human decidualization needs to be established. In order to achieve a better understanding of the E-regulated pathways underlying this important physiological process, it is necessary to identify gene networks that operate under the control of ESR1. Therefore, one of the major aims of my project is to analyze the gene networks controlled by ESR1 during human stromal cell differentiation.

The requirement of progesterone receptor for decidualization has been confirmed by many studies including the PRA knockout mouse model, which is impaired in decidualization [31]. *In vitro* studies in human stromal cells confirmed the essential role of progesterone receptor in human decidualization [22]. Both isoforms of the progesterone receptor, PRA and PRB, are expressed in the stromal cells. The relative levels of the isoforms change during the menstrual cycle. During the early proliferative stage, PRB level is very low, while PRA levels are high. During the peri-ovulatory period, expression of both isoforms increase, and the levels of PRB becomes comparable to PRA levels. By late secretory phase, PRB levels decline [32, 33]. Some endometrial disorders show aberrant expression and relative ratios of PRA and PRB. For example, it was reported that the PRA:PRB ratio was abnormal in endometrial cancer [34, 35] and endometriosis [36], underlining the importance of distinguishing between the roles of these isoforms. Although, the molecular pathways via which the progesterone receptors exert their

effects during decidualization have been addressed in some studies [37-39], we are still far away from understanding the downstream signaling mechanisms of each isoform during decidualization in the human. A central aim of my project is to analyze the gene networks controlled by PRA and PRB during human endometrial differentiation. A better understanding of these gene networks will help identify the mechanisms underlying the normal uterine function and abnormalities in endometrial disorders and offer potential new targets for therapeutic studies.

Role of cAMP-dependent signaling during decidualization

Numerous previous studies have documented that cAMP-induced signaling pathways are important in decidualization [22, 40, 41]. P alone or in combination with E is a very weak inducer of *in vitro* decidualization of HESCs [42]. Peptide hormones, such as LH/hCG, CRH, and relaxin, and prostanoids, such as PGE2, contribute to decidual transformation *in vitro* by binding to their G protein coupled receptors and increasing intracellular cAMP levels [43-46]. Studies have shown that human endometrial stromal cells express the receptors of these hormones [47-49]. Studies have shown that the level of intracellular cAMP is increased in stromal cells during decidualization *in vivo* [50, 51]. However, how intracellular cAMP levels are regulated by these hormones during decidualization is not well understood. Cyclic AMP is an important second messenger molecule which is generated upon binding of a ligand to its G-protein coupled receptor (GPCR). Ligand binding induces a conformational change, leading to the release of the G alpha subunit from the trimeric G alpha-beta-gamma complex. G alpha subunit regulates the activity of adenylyl cyclase enzyme which produces cAMP from ATP [52]. Three major targets of cAMP signaling are protein kinase A (PKA), GTP-exchange protein (EPAC) and the cyclic nucleotide gated ion channels [53], with PKA being the predominant downstream target molecule [54]. In its inactive state, PKA is composed of two regulatory and two catalytic subunits. When two cAMP molecules bind to each regulatory subunit, a conformational change takes place along with the release of catalytic subunits from the regulatory subunits. The free and active catalytic subunits are able to phosphorylate target molecules in the cytoplasm as well as in the nucleus [54]. Several proteins have been identified

as substrates of PKA, including transcription factors (CREB, CREM, and ATF-1), metabolic enzymes and cytoskeletal elements [53, 55].

Although cAMP analogs are widely used during *in vitro* decidualization of HESCs, the molecular targets of this signaling during this differentiation process are mostly unknown. It is conceivable that PKA, upon activation by cAMP, phosphorylates downstream regulatory molecules that play important roles in decidualization. Post-translational modification via phosphorylation is an important and well-studied mechanism of regulation. Therefore, identification of targets of phosphorylation by PKA during HSC decidualization is necessary to understand the mechanisms by which cAMP signaling impacts on this process. Hence, we investigated the role of cAMP signaling during decidualization process by identifying the phosphorylated targets of cAMP-activated PKA and evaluating their functions during this process.

REFERENCES

1. Leitao, B., et al., *Silencing of the JNK pathway maintains progesterone receptor activity in decidualizing human endometrial stromal cells exposed to oxidative stress signals*. FASEB J, 2010. **24**(5): p. 1541-51.
2. Brosens, J.J. and B. Gellersen, *Death or survival--progesterone-dependent cell fate decisions in the human endometrial stroma*. J Mol Endocrinol, 2006. **36**(3): p. 389-98.
3. Maruyama, T., et al., *Human uterine stem/progenitor cells: their possible role in uterine physiology and pathology*. Reproduction, 2010. **140**(1): p. 11-22.
4. Wang, H. and S.K. Dey, *Roadmap to embryo implantation: clues from mouse models*. Nat Rev Genet, 2006. **7**(3): p. 185-99.
5. Carson, D.D., et al., *Embryo implantation*. Dev Biol, 2000. **223**(2): p. 217-37.
6. Ramathal, C.Y., et al., *Endometrial decidualization: of mice and men*. Semin Reprod Med, 2010. **28**(1): p. 17-26.
7. Salker, M., et al., *Natural selection of human embryos: impaired decidualization of endometrium disables embryo-maternal interactions and causes recurrent pregnancy loss*. PLoS One, 2010. **5**(4): p. e10287.
8. Brosens, J.J., et al., *A role for menstruation in preconditioning the uterus for successful pregnancy*. Am J Obstet Gynecol, 2009. **200**(6): p. 615 e1-6.
9. Brosens, J.J., R. Pijnenborg, and I.A. Brosens, *The myometrial junctional zone spiral arteries in normal and abnormal pregnancies: a review of the literature*. Am J Obstet Gynecol, 2002. **187**(5): p. 1416-23.
10. Gellersen, B., I.A. Brosens, and J.J. Brosens, *Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives*. Semin Reprod Med, 2007. **25**(6): p. 445-53.
11. Beato, M., *Gene regulation by steroid hormones*. Cell, 1989. **56**(3): p. 335-44.

12. Katzenellenbogen, B.S., et al., *Structure-function relationships in estrogen receptors and the characterization of novel selective estrogen receptor modulators with unique pharmacological profiles*. Ann N Y Acad Sci, 2001. **949**: p. 6-15.
13. Chang, E.C., et al., *Estrogen Receptors alpha and beta as determinants of gene expression: influence of ligand, dose, and chromatin binding*. Mol Endocrinol, 2008. **22**(5): p. 1032-43.
14. Kastner, P., et al., *Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B*. EMBO J, 1990. **9**(5): p. 1603-14.
15. Lydon, J.P., et al., *Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities*. Genes Dev, 1995. **9**(18): p. 2266-78.
16. Franco, H.L., et al., *In vivo analysis of progesterone receptor action in the uterus during embryo implantation*. Semin Cell Dev Biol, 2008. **19**(2): p. 178-86.
17. Satokata, I., G. Benson, and R. Maas, *Sexually dimorphic sterility phenotypes in Hoxa10-deficient mice*. Nature, 1995. **374**(6521): p. 460-3.
18. Mulac-Jericevic, B. and O.M. Conneely, *Reproductive tissue selective actions of progesterone receptors*. Reproduction, 2004. **128**(2): p. 139-46.
19. Hewitt, S.C., J.C. Harrell, and K.S. Korach, *Lessons in estrogen biology from knockout and transgenic animals*. Annu Rev Physiol, 2005. **67**: p. 285-308.
20. Xu, J., et al., *Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene*. Science, 1998. **279**(5358): p. 1922-5.
21. Park, S., et al., *Uterine development and fertility are dependent on gene dosage of the nuclear receptor coregulator REA*. Endocrinology, 2012. **153**(8): p. 3982-94.
22. Gellersen, B. and J. Brosens, *Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair*. J Endocrinol, 2003. **178**(3): p. 357-72.

23. Klemmt, P.A.B., et al., *Stromal cells from endometriotic lesions and endometrium from women with endometriosis have reduced decidualization capacity*. Fertil Steril, 2006. **85**(3): p. 564-572.
24. Tang, B., S. Guller, and E. Gurpide, *Mechanisms involved in the decidualization of human endometrial stromal cells*. Acta Eur Fertil, 1993. **24**(5): p. 221-3.
25. Irwin, J.C., et al., *Hormonal regulation of human endometrial stromal cells in culture: an in vitro model for decidualization*. Fertil Steril, 1989. **52**(5): p. 761-8.
26. Buzzio, O.L., et al., *FOXO1A differentially regulates genes of decidualization*. Endocrinology, 2006. **147**(8): p. 3870-6.
27. Aghajanova, L., et al., *Steroidogenic enzyme and key decidualization marker dysregulation in endometrial stromal cells from women with versus without endometriosis*. Biol Reprod, 2009. **80**(1): p. 105-14.
28. Mylonas, I., et al., *Immunohistochemical analysis of estrogen receptor alpha, estrogen receptor beta and progesterone receptor in normal human endometrium*. Acta Histochem, 2004. **106**(3): p. 245-52.
29. Lecce, G., et al., *Presence of estrogen receptor beta in the human endometrium through the cycle: expression in glandular, stromal, and vascular cells*. J Clin Endocrinol Metab, 2001. **86**(3): p. 1379-86.
30. Zhao, Y., et al., *The coregulator, repressor of estrogen receptor activity (REA), is a crucial regulator of the timing and magnitude of uterine decidualization*. Endocrinology, 2013. **154**(3): p. 1349-60.
31. Mulac-Jericevic, B. and O.M. Conneely, *Reproductive tissue-selective actions of progesterone receptors*. Ernst Schering Res Found Workshop, 2005(52): p. 19-37.
32. Mote, P.A., et al., *Colocalization of progesterone receptors A and B by dual immunofluorescent histochemistry in human endometrium during the menstrual cycle*. J Clin Endocrinol Metab, 1999. **84**(8): p. 2963-71.

33. Mangal, R.K., et al., *Differential expression of uterine progesterone receptor forms A and B during the menstrual cycle*. J Steroid Biochem Mol Biol, 1997. **63**(4-6): p. 195-202.
34. Kumar, N.S., et al., *Selective down-regulation of progesterone receptor isoform B in poorly differentiated human endometrial cancer cells: implications for unopposed estrogen action*. Cancer Res, 1998. **58**(9): p. 1860-5.
35. Sasaki, M., et al., *Progesterone receptor B gene inactivation and CpG hypermethylation in human uterine endometrial cancer*. Cancer Res, 2001. **61**(1): p. 97-102.
36. Attia, G.R., et al., *Progesterone receptor isoform A but not B is expressed in endometriosis*. J Clin Endocrinol Metab, 2000. **85**(8): p. 2897-902.
37. Rubel, C.A., et al., *Research resource: Genome-wide profiling of progesterone receptor binding in the mouse uterus*. Mol Endocrinol, 2012. **26**(8): p. 1428-42.
38. Cloke, B., et al., *The androgen and progesterone receptors regulate distinct gene networks and cellular functions in decidualizing endometrium*. Endocrinology, 2008. **149**(9): p. 4462-74.
39. Popovici, R.M., L.C. Kao, and L.C. Giudice, *Discovery of new inducible genes in in vitro decidualized human endometrial stromal cells using microarray technology*. Endocrinology, 2000. **141**(9): p. 3510-3.
40. Telgmann, R., et al., *Activated protein kinase A is required for differentiation-dependent transcription of the decidual prolactin gene in human endometrial stromal cells*. Endocrinology, 1997. **138**(3): p. 929-937.
41. Jones, M.C., et al., *Regulation of the SUMO pathway sensitizes differentiating human endometrial stromal cells to progesterone*. Proc Natl Acad Sci U S A, 2006. **103**(44): p. 16272-7.
42. Brosens, J.J., N. Hayashi, and J.O. White, *Progesterone receptor regulates decidual prolactin expression in differentiating human endometrial stromal cells*. Endocrinology, 1999. **140**(10): p. 4809-20.

43. Frank, G.R., et al., *Prostaglandin E2 enhances human endometrial stromal cell differentiation*. Endocrinology, 1994. **134**(1): p. 258-63.
44. Ferrari, A., F. Petraglia, and E. Gurbide, *Corticotropin releasing factor decidualizes human endometrial stromal cells in vitro. Interaction with progestin*. J Steroid Biochem Mol Biol, 1995. **54**(5-6): p. 251-5.
45. Tseng, L., et al., *Effect of progestin, antiprogestin, and relaxin on the accumulation of prolactin and insulin-like growth factor-binding protein-1 messenger ribonucleic acid in human endometrial stromal cells*. Biol Reprod, 1992. **47**(3): p. 441-50.
46. Tang, B. and E. Gurbide, *Direct effect of gonadotropins on decidualization of human endometrial stroma cells*. J Steroid Biochem Mol Biol, 1993. **47**(1-6): p. 115-21.
47. Han, S.W., Z.M. Lei, and C.V. Rao, *Homologous down-regulation of luteinizing hormone/chorionic gonadotropin receptors by increasing the degradation of receptor transcripts in human uterine endometrial stromal cells*. Biol Reprod, 1997. **57**(1): p. 158-64.
48. Milne, S.A., et al., *Expression, localization, and signaling of PGE(2) and EP2/EP4 receptors in human nonpregnant endometrium across the menstrual cycle*. J Clin Endocrinol Metab, 2001. **86**(9): p. 4453-9.
49. Di Blasio, A.M., et al., *Expression of corticotropin-releasing hormone and its R1 receptor in human endometrial stromal cells*. J Clin Endocrinol Metab, 1997. **82**(5): p. 1594-7.
50. Pansini, F., et al., *Sex steroid hormones influence the cAMP content in human endometrium during the menstrual cycle*. Gynecol Obstet Invest, 1984. **18**(4): p. 174-7.
51. Maruyama, T. and Y. Yoshimura, *Molecular and cellular mechanisms for differentiation and regeneration of the uterine endometrium*. Endocr J, 2008. **55**(5): p. 795-810.
52. Sunahara, R.K., C.W. Dessauer, and A.G. Gilman, *Complexity and diversity of mammalian adenylyl cyclases*. Annu Rev Pharmacol Toxicol, 1996. **36**: p. 461-80.

53. Fimia, G.M. and P. Sassone-Corsi, *Cyclic AMP signalling*. J Cell Sci, 2001. **114**(Pt 11): p. 1971-2.
54. Skalhogg, B.S. and K. Tasken, *Specificity in the cAMP/PKA signaling pathway. Differential expression, regulation, and subcellular localization of subunits of PKA*. Front Biosci, 2000. 5: p. D678-93.
55. Howe, A.K., *Regulation of actin-based cell migration by cAMP/PKA*. Biochim Biophys Acta, 2004. **1692**(2-3): p. 159-74.

CHAPTER II

ROLE OF ESR1 AND cAMP DURING HUMAN ENDOMETRIAL STROMAL CELL DIFFERENTIATION

ABSTRACT

The differentiation of endometrial stromal cells, known as decidualization, is a prerequisite for a successful pregnancy. Estrogen and progesterone, whose actions are mediated by their intracellular receptors, are critical regulators of this differentiation process. The goal of this study is to determine the role of estrogen receptor alpha (ESR1) in human endometrial stromal cell (HESC) differentiation by exploring the mechanisms underlying its functions. Using a well-established *in vitro* differentiation model of primary cultures of HESC, we showed that ESR1 was required for HESC differentiation. Gene expression profiling experiments indicated that ESR1 regulated HESC differentiation in two ways: (i) by suppressing cell proliferation; (ii) by promoting differentiation through regulating the expression of critical players of HESC differentiation including *PGR*, *FOXO1* and *WNT4*. Interestingly, expressions of these targets were dependent on the presence of 8-Br-cAMP, suggesting a link between cAMP and ESR1 signaling. Using a proteomic approach, we identified MED1, a subunit of the Mediator coactivator complex, as a target of PKA in differentiating HESC.

We found that MED1 interacted with ESR1 in HESC and silencing MED1 expression severely impaired stromal differentiation. In the presence of 8-Br-cAMP, the interaction between ESR1 and MED1 was increased which coincided with the increased recruitment of MED1 to the ESR1 binding regions of *WNT4* gene and increased *WNT4* expression. We conclude that ESR1 plays a critical role in HESC differentiation by suppressing proliferation and inducing the expression of critical regulators. cAMP signaling modulates transcriptional activity of ESR1 by phosphorylating ESR1 coactivator MED1.

INTRODUCTION

Decidualization is a complex transformation process by which the endometrial tissue becomes competent to promote embryo-uterine interactions which is essential for a successful embryo implantation and maintenance of pregnancy [1-3]. The human endometrium first

undergoes an E-dominated proliferative phase followed by a P-dominated secretory phase [4] during which decidualization of endometrial stromal cells, termed predecidualization, occurs independent of conception. If there is no conception, the circulating P levels go down in the late secretory phase, leading to the shedding of the decidualized tissue layer, triggering menstruation. If pregnancy occurs, decidualization progresses further and spreads through the endometrium, helping trophoblast invasion and placenta formation [5]. Impaired decidualization is associated with endometrial and reproductive diseases and dysfunctions, such as menstrual disorders, endometriosis, infertility and early pregnancy loss [6, 7]. Therefore, a better understanding of the decidualization process will give valuable information to address the underlying deficiencies in some of the endometrial disorders.

E and P act mainly through their nuclear receptors. Two receptor subtypes have been identified for estrogen: estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2) [8]. Estrogen receptor alpha knock out female mouse is infertile and defective in decidualization (unpublished data). Estrogen receptor beta null mouse, which is subfertile due to ovarian defects, is reported to retain normal uterine functions including decidualization and can support normal pregnancy [9]. Additionally, the steroid receptor coactivator-1 null females exhibit defects in decidualization [10]. These *in vivo* data support the importance of ESR1 in regulation of decidualization process. However, the molecular mechanisms downstream of it are still not well understood.

In order to study the molecular pathways underlying decidualization in human, an *in vitro* system using primary cultures of proliferative phase HESC are used. It is shown that these primary cultures retain their biological characteristics in early passages and exhibit a decidualization phenotype that is very similar to endometrial stroma *in vivo* at both biochemical and morphological levels [11, 12]. Addition of a differentiation cocktail composed of E, P, and a cyclic AMP analog 8-Br-cAMP triggers differentiation of HESCs [13-15]. The fibroblastic stromal cells undergo a distinct morphological change as they are transformed into larger, and round decidual cells. This transformation in the cellular architecture is accompanied by a dramatic rise in the expression of known biomarkers of decidualization. A number of biochemical markers, such as prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP1), have been widely used to monitor the progress of the decidualization program *in vitro*

[11, 16]. Numerous previous studies have documented that cAMP-induced signaling pathways are important in decidualization [11, 17, 18]. P alone or in combination with E is a very weak inducer of *in vitro* decidualization of HESCs [19]. It has also been reported that the intracellular cAMP levels were increased in stromal cells during decidualization *in vivo* [20, 21]. Cyclic AMP is an important second messenger with PKA being its predominant downstream target molecule [22]. Several proteins have been identified as substrates of PKA, including transcription factors (CREB, CREM and ATF-1), metabolic enzymes and cytoskeletal elements [23, 24]. Although cAMP analogs are widely used during *in vitro* decidualization of HESCs, the molecular targets of this signaling during the differentiation process are mostly unknown.

In order to achieve a better understanding of the E-regulated pathways underlying this human stromal cell differentiation, we identified gene networks that operate under the control of ESR1. This study aimed to address the role of cAMP signaling as well.

MATERIALS AND METHODS

Cell Line and Cell Culture

The primary human endometrial stromal cells (HESC) were provided by Dr. Robert Taylor of Emory University Medical School. HESC were isolated from biopsies collected from proliferative stage endometrium of normal cycling women with no sign of uterine abnormality and with the volunteer's informed consent. These cells were cultured in DMEM/F-12 medium (Invitrogen) supplemented with 5% (v/v) fetal bovine serum (Hyclone), 50µg/ml penicillin and 50µg/ml streptomycin (Invitrogen). HESC of passage number nine or earlier were used in this study. For *in vitro* differentiation, the cells were treated with differentiation cocktail composed of 0.5mM 8-bromo-adenosine-3',5'-cyclic monophosphate (8-Br-cAMP) (Sigma), 1µM progesterone (Sigma) and 10nM 17-β-estradiol (Sigma) in DMEM/F-12 medium (Invitrogen) supplemented with 2% (v/v) charcoal dextran stripped fetal bovine serum. The medium was refreshed in every 48h.

siRNA transfection

HESC were transfected with siRNA targeting *ESR1* (Ambion), *PGR* (Dharmacon), *PRKACA* (Dharmacon), *MED1* (Dharmacon) or non-targeting control siRNA (Ambion/Dharmacon) following manufacturer's protocol (SilentFect, Bio-Rad Laboratories). To downregulate expressions of *ESR1*, *PGR* and *MED1*, a final siRNA concentration of 20nM and to downregulate *PRKACA*, a final concentration of 40nM siRNA were used. Briefly, proper dilution of siRNA was mixed together with SilentFect (5µl per reaction) to transfect the cells. At 48h following siRNA treatment, medium was removed, cells were washed with PBS and cells were treated with differentiation cocktail for time periods as indicated in the figure legends.

Double thymidine block

Double thymidine block method was used with minor modifications to synchronize HESC [25]. Briefly, cells were seeded in 2-well chamber slides. Cells were grown to 60% confluency, and then cells were washed with 1xPBS and incubated in growth medium with 2mM thymidine for 12-14h. Thymidine block was released by removing thymidine containing medium and washing cells with 1xPBS three times. Cells were incubated in fresh growth medium for 12h. The siRNA was added to the cells during the release and the transfection was carried out for 24h to 36h concurrent with the second thymidine block which was started by adding thymidine directly to siRNA containing medium to a final concentration of 2nM. Thymidine and siRNA treatment was removed and cells were washed with 1xPBS three times; then cells were incubated in differentiation cocktail containing medium. To monitor cell proliferation, we added BrdU to the medium during the differentiation cocktail treatment.

Immunocytochemistry

HESC were fixed in 4% formalin solution (Sigma) at room temperature for 10 min, followed PBS washes. The cells were permeabilized by 0.1% Triton X-100 in PBS for 15 min and non-specific binding of antibodies was blocked with 10% normal donkey serum at room temperature for 1 h. Alexa Fluor 488 phalloidin (Invitrogen), which selectively recognizes F-actin, was used at a dilution of 1:50 and cells were incubated with the antibody for 20 min at room temperature. DAPI (1µg/ml) was used as counter staining.

Microarray sample preparation and data analysis

HESC were transfected with siRNA targeting *ESR1* (Ambion) or non-targeting control siRNA (Ambion) as explained above. Total RNA was isolated by Trizol reagent (Invitrogen, CA) and further purified using RNeasy columns (Qiagen) following manufacturer's instructions. RNA samples were processed at the Biotechnology Center of the University of Illinois at Urbana-Champaign. RNA integrity was verified using Agilent 2100 Bioanalyzer. Each RNA sample was processed to generate labeled cRNA following established protocols for hybridization to the GeneChip Human Gene 1.0 ST arrays, which offer whole-transcript coverage by representing each of the 28,869 genes [26]. The resulting data files were analyzed by Affymetrix GeneChip Expression Console software using RMA algorithm. Genes with a minimum of 1.2-fold-change in the same direction in all three independent microarrays were considered as differentially regulated genes. Canonical pathways analysis identified the pathways from the Ingenuity Pathways Analysis library of canonical pathways (Ingenuity Systems) [27] which were most significantly enriched in the data set. Subsets of the related genes that were assigned to a canonical pathway were considered for further analysis.

RNA Isolation and Real-time PCR Analysis

Total RNA was extracted from cultured HESC using Trizol (Invitrogen) according to manufacturer's instructions. Total RNA was subjected to cDNA conversion using Stratagene cDNA conversion kit, following manufacturer's instructions. cDNA were subjected to quantitative PCR analysis using gene specific primers. 36B4 was used as the internal control. For a given sample, threshold cycle (Ct) and (standard deviation) SD was calculated from individual Ct values from three technical replicates. Normalized mean Ct (ΔCt) was calculated by subtracting mean Ct of 36B4 from mean Ct of a target gene for control sample. $\Delta\Delta Ct$ was then calculated for each gene as a difference in ΔCt values between control and sample. Fold change in gene expression was then computed as $2^{-\Delta\Delta Ct}$. The RNA samples were prepared from minimum of three separate primary cultures subjected to the same experimental treatment. The real time PCR results are expressed as mean $\pm$ standard deviation of three biological replicates. Statistical significance of the data was determined using student t-test. A p-value of <0.05 was considered significant.

Adenovirus Transduction

HESC were transduced with the adenovirus expressing Flag tagged ESR1 for 24h before the treatments. The flag-tagged-ESR1 expressing adenovirus was kindly provided by laboratory of Prof. Benita Katzenellenbogen of University of Illinois at Urbana-Champaign.

Western Blot Analysis

Primary HESCs were subjected to different treatments as indicated in the figure legends for indicated periods of time. Whole cell extracts were prepared and equal amounts of proteins were analyzed by SDS-PAGE, transferred to PVDF membrane and the specific proteins were detected by Western blotting using the antibody against Calnexin (Santa Cruz), ESR1 and antibodies against phospho-ESR1: Ser118 (Phospho-Estrogen Receptor α Antibody Sampler Kit, Cell Signaling, #9924). Antibody recognizing PGR was kindly provided by Prof. Dean Edwards of Baylor College of Medicine. Phospho-PKA Substrate (RRXS*/T*) (100G7E) Rabbit mAb #9624 was purchased from Cell Signaling. PKA is a member of AGC kinase subfamily which recognizes substrates by arginine at position -3 relative to the phosphorylated serine or threonine [28]. The antibody was raised against KLH-coupled synthetic peptides, known as phospho-PKA substrate (RRXS/T). It can efficiently recognize proteins containing a phosphorylated serine or threonine residue with arginine at the -3 and -2 positions whereas it does not recognize the nonphosphorylated form [29]. Two antibodies against MED1 were used (A300-793A, Bethyl Laboratories; sc8998, Santa Cruz Biotechnology).

Immunoprecipitation and Silver Staining

Primary HESCs were subjected to decidualization *in vitro* in the presence of differentiation cocktail and the cells were harvested at different times after cocktail addition. Whole cell extracts were prepared and equal amounts of proteins were used for immunoprecipitation (IP). IP was performed using Direct IP Kit following manufacturer's instructions (Thermo Scientific). Immune complexes eluted were separated by SDS-PAGE. The gel was silver stained using Silver Quest Staining kit (Invitrogen) following manufacturer's instructions. Specific bands cut from the gel were submitted to Proteomics Center of UIUC for LC/MS analysis for protein identification.

Chromatin immunoprecipitation

ChIP assays were performed using the EZ-ChIP kit (Millipore) according to the manufacturer's instructions with minor modifications. HESC were seeded on 150mm dishes. After cells were attached, medium was switched to 2% (v/v) charcoal dextran stripped fetal bovine serum containing medium and transduced with adenovirus carrying flag-tagged ESR1 for 24h. Next day, cells were either treated with E+P or E+P+8-Br-cAMP for 30 min. Then, cells were fixed with 1% formaldehyde for 10 min and excess formaldehyde was quenched with 0.25 M glycine for 5 min. Cells were then washed twice with cold PBS, scraped and collected in PBS containing protease inhibitors in a canonical tube and centrifuged. Cell pellet was resuspended in lysis buffer containing protease inhibitors for 15 min to lyse the cells. Next, chromatin was sonicated in four 15-s pulses with cooling between pulses (Fisher Scientific model 100 Sonic Dismembrator). One percent of the cell lysate was used for input control, and the rest was incubated with anti-flag M2 affinity gel (Sigma, A2220) to immunoprecipitate flag-ESR1 or anti-MED1 antibody (Santa Cruz, sc-8998) overnight at 4⁰C to isolate the immune complexes. Normal mouse IgG (Santa Cruz, sc-2027) immunoprecipitation served as a negative control. Complexes were washed with low salt, high salt, LiCl buffers and twice with TE buffers, eluted from the beads and heated at 65°C for 6 hours to reverse the cross-linking. After digestion with RNAase A and proteinase K, DNA fragments were purified using QIAquick PCR purification kit (Qiagen). For ChIP, real-time quantitative PCR (Q-PCR) primers were designed to amplify the potential ESR1 binding element (ERE) in the regulatory regions of *WNT4* gene and also a primer pair flanking an ERE free sequence was used as a negative control. The resulting signals were normalized to input DNA and relative fold enrichment was calculated as the enrichment in experimental samples over that of negative control ChIP.

RESULTS

ESR1 is required for HESC differentiation

In vivo studies have shown that ESR1 is expressed in human endometrial stroma during the proliferative and secretory phases of the menstrual cycle [30]. We monitored ESR1 expression in

primary cultures of HESC isolated from endometrial biopsies collected from early proliferative phase uterus. We detected *ESR1* mRNA and ESR1 protein in the proliferative stage stromal cells prior to the addition of a differentiation cocktail containing E, P, and 8-Br-cAMP. Addition of the differentiation cocktail, which induced decidualization of HESC, led to a marked increase in ESR1 mRNA and protein levels within 24 h (Fig. 2.1, A and B). It is known that the estrogen receptor is phosphorylated at several residues and its phosphorylation status affects its transcriptional activity. It is generally accepted that phosphorylation at Ser118 enhances transcriptional activity of the receptor by increasing the recruitment of coactivators [31]. Therefore, we analyzed the Ser118 phosphorylation of ESR1 in differentiating HESC compared to undifferentiated cells. For easy isolation of ESR1 and assessment of its phosphorylation state, we enhanced the level of ESR1 in HESC by expressing a flag-tagged human ESR1 in these cells via an adenoviral vector. 24h following viral transduction, HESC were treated with or without the differentiation cocktail for 6 h. Ser-118 phosphorylation was very low in the undifferentiated cells and significantly enhanced upon addition of the differentiation cocktail (Fig. 2.1C). The enhanced expression of ESR1 and a distinct change in its phosphorylation during the initiation of the decidualization program hinted at a potential role of this ligand-inducible transcription factor in HESC differentiation. To test this possibility, ESR1 expression was silenced in HESC by administering siRNA specifically targeting ESR1 mRNA and then we examined the effect of this down regulation on the differentiation process. Analysis of mRNAs corresponding to PRL and IGFBP1, well-known biochemical markers of decidualization, by real-time qPCR revealed a significant decrease in the expression of these genes (Fig. 2.1D). These gene expression changes, indicating a lack of stromal differentiation in the absence of ESR1, were also consistent with the lack of morphological changes that one associated with decidualization process (Fig. 2.1E). HESC displayed the round epitheloid characteristics of the differentiated cells in the presence of ESR1, but failed to acquire this morphology and remained fibroblastic in the absence of this receptor. Collectively, these results supported our view that ESR1 plays a critical regulatory role in the *in vitro* differentiation of HESC.

Cell cycle progression is suppressed downstream of ESR1 during HESC differentiation

To identify the ESR1-regulated downstream pathways associated with HESC differentiation, we used oligonucleotide microarrays. Our approach involved comparing the gene expression

profiles of three different clinical samples of proliferative phase HESC transfected with siRNA targeted to *ES1* mRNA or non-targeting control siRNA as described in materials and methods section. When the gene expression profiles of control and ESR1-depleted samples of three independent sample sets were compared, 194 genes were found to be commonly down-regulated while 318 genes were up-regulated. Genes with altered regulation were classified according to their known biological functions and the canonical pathways using Ingenuity Pathway Analysis (Table 2.1). A prominent category of genes whose expression was altered in the absence of ESR1 was cell cycle and growth regulatory pathways. The expressions of the cycle regulatory molecules, including *CCNE2*, *CCNA2*, *CDC20*, *CDK1*, *CDK2*, *PLK4* and *SKP2* were upregulated in ESR1-depleted HESC. We also noted that important suppressors of cell proliferation, including *CDKN1C* (cyclin dependent kinase inhibitor 1C or p57) and *GAS1* (growth arrest specific 1) were downregulated in the absence of ESR1 (Table 2.2). These results indicated that ESR1 played cell cycle regulatory functions during HESC differentiation. It is generally observed that the cell cycle exit is a pre-requisite for differentiation [32]. We and others have observed that HESC, which showed robust proliferation in the growth medium, ceased proliferation under differentiation conditions (Fig. 2.2A); suggesting that the exit from the cell cycle is a key step in HESC differentiation and ESR1 may be involved in this process. To validate the altered expression of the microarray-derived cell cycle regulatory genes downstream of ESR1, we performed real-time PCR analysis. We confirmed that the expressions of a subset of cell cycle related genes identified in the microarray studies including *CCNE2*, *CCNA2*, *CDC20* and *CDK1*, were upregulated in ESR1-depleted HESC, while that of *CDKN1C* and *GAS1* was downregulated in the absence of this receptor. We also found that the expression levels of *E2F1* and *E2F2* were upregulated in HESC when ESR1 was down-regulated, whereas *E2F3* levels remained unchanged (Fig. 2.2B). Since the expression of genes which are critical regulators of the G1-S transition was altered in the absence of ESR1, we considered the possibility that ESR1 suppresses this critical step of the cell cycle during HESC differentiation. Next, we investigated whether the loss of ESR1 actually resulted in the stimulation of cell cycle progression of HESC under the differentiation conditions as suggested by the gene expression changes shown in Fig. 2.2B. To test this possibility, we first synchronized the HESC at the G1/S boundary of the cell cycle, using double thymidine block method [25]. Our results indicated a significant increase in BrdU incorporation in HESC depleted of ESR1 compared to the control samples (Fig. 2.2C).

Collectively, these results suggest that ESR1 suppresses the cell cycle at G1/S phase during differentiation of HESC.

Well-known regulators of decidualization are controlled by ESR1

Among the many genes whose expression was down-regulated in the gene expression profile in response to the attenuation of ESR1 expression, we found three important known regulators of HESC differentiation: progesterone receptor (PGR), Forkhead Box Protein O1 (FOXO1) and wingless-type MMTV integration site family, member 4 (WNT4) [33-35]. As shown in Fig. 2.3A, expressions of both *FOXO1* and *WNT4* mRNAs were markedly downregulated at 24h and 72 h of differentiation when ESR1 expression was silenced. We did not detect a significant down-regulation of *PGR* mRNA at 24h of differentiation. However, *PGR* mRNA levels were markedly down regulated in ESR1-depleted cells at 72h of differentiation (Fig. 2.3A). These results are consistent with a role of ESR1 as a regulator of HESC differentiation. Since the *PGR* levels did not change significantly while that of *FOXO1* and *WNT4* levels was markedly diminished in ESR1-depleted HESC at 24 h of differentiation cocktail treatment, we hypothesized that *FOXO1* and *WNT4* are regulated by ESR1 in a PGR independent manner. Real-time PCR analysis confirmed that *FOXO1* expression was downregulated specifically by siRNA mediated silencing of ESR1 but not PGR (Fig. 2.3B). Similarly, FOXO1 protein level was markedly reduced in ESR1-depleted HESC while it did not change upon PGR knock down (Fig 2.3C), suggesting that FOXO1 was a downstream target of ESR1 during decidualization of HESC and its regulation by ESR1 occurred in a PGR-independent manner. It is noteworthy that FOXO1 might be the key downstream molecule under ESR1 which suppresses stromal cell proliferation during differentiation, as FOXO1 was shown to inhibit G1-S transition of HESC under differentiation conditions [35].

Expression of ESR1 target genes in differentiating HESC requires addition of cAMP

After identifying *FOXO1*, *WNT4* and *PGR* as downstream targets of ESR1, we monitored their expressions in response to E treatment. To our surprise, we did not detect significant up-regulation of these genes when HESC were treated with estrogen alone or estrogen plus progesterone while the addition of cAMP to the cocktail led to a robust increase in their expression (Fig. 2.3D). We should note that the expression of these genes is ESR1 dependent

since their expression is impaired when the receptor is silenced in the presence of cAMP (Figures 2.3A and 2.3B), suggesting that there might be a link between cAMP and ESR1 signaling to regulate the expression of these genes.

PKA signaling is required for *in vitro* differentiation of HESC

The cyclic AMP analogue, 8-Br- cAMP, used for *in vitro* differentiation of HESC can activate both PKA and EPAC [36]. Gellersen and Brosens reported that treatment of undifferentiated HESC with a novel EPAC-specific cAMP analogue -8-p CPT-2_*O*-methyl-cAMP- [37], did not induce a decidual phenotype [11], suggesting that PKA is the critical target of cAMP for induction of differentiation of HESC. In support of this, it has been reported that decidualization is impaired when HESC are treated with PKA inhibitor H89 [38]. However, there are several reports documenting that H89 has unspecific inhibitory effects on other kinases [39]. Therefore, we wanted to confirm that PKA is the principal target of cAMP during *in vitro* differentiation of HESCs by using a more specific tool. We used siRNA approach to knock down the major catalytic subunit of PKA, alpha (PRKACA), in HESC [17] and monitored the expression of well-known decidual markers in PRKACA-depleted cells. The cells treated with PRKACA-specific siRNA showed a marked impairment in expression of decidualization markers compared to control siRNA treatment (Fig. 2.4A). These results suggested that PKA signaling, when activated upon binding to cAMP, is critical for induction of HESC differentiation.

MED1 is a downstream target of cAMP signaling during HESC differentiation

It is conceivable that cAMP activates PKA and active PKA phosphorylates downstream regulatory molecules that play important roles in decidualization. To identify the targets of PKA-dependent phosphorylation during decidualization, we employed a monoclonal antibody that specifically recognizes the phosphorylated PKA substrates. To determine the pattern of PKA-dependent phosphorylation in response to cAMP, HESCs were untreated or treated with E+P or E+P+cAMP. The cells were harvested at 24h after cocktail addition. Whole cell extracts were prepared and equal amounts of proteins were analyzed by SDS-PAGE. The phosphorylated PKA target proteins were detected by Western blotting using the antibody against phospho-PKA substrate. A number of phosphorylated bands were already present in extracts of untreated cells.

We did not observe prominent changes in E+P treated sample. Strikingly, several additional bands appeared in the sample treated with E+P+cAMP (Fig. 2.5A). To further ascertain that the phosphorylated bands were generated by actions of PKA, we treated the cultures with H89, a PKA inhibitor. In the presence of H89, the majority of the phosphorylated bands were reduced in intensity or absent, confirming that they represented PKA targets (Fig. 2.5B) and we were able to get similar results when we silenced the expression of the catalytic subunit of PKA with siRNA and (data not shown). These results confirmed that the antibody could specifically detect phosphorylated targets of PKA. To identify these phosphorylated targets of PKA in differentiating HESC, whole cell protein extracts were prepared from HESCs treated with or without differentiation cocktail for 24 h. The phosphorylated proteins were immunoprecipitated (IP) using the phospho-PKA substrate antibody. The immune complexes were isolated and analyzed by SDS PAGE and the banding pattern was visualized by silver staining the gel (Fig. 2.5C). The banding patterns of PKA-phosphorylated polypeptides in untreated and differentiation cocktail-treated were compared and individual bands with increased intensity were cut out and subjected to analysis by Mass Spectrometry (LC/MS). Among potential targets including vimentin, non-muscle myosin 9, skeletal tropomyosin and MED1 (Fig. 2.5D), we decided to focus on MED1 which is a subunit of the Mediator complex that forms a bridge between the transcription factors and the general transcription machinery [40] and by *in vitro* studies, it has been shown to be a target of several kinases including MAPK, PKC and PKA [41]. To further confirm that MED1 is phosphorylated by PKA in differentiating HESC, we immunoprecipitated MED1 with a MED1 specific antibody from whole cell extracts of either untreated or differentiation cocktail treated HESC. We then performed a western blot analysis with phospho-PKA substrate antibody. In the extracts derived from untreated cells, we detected a faint band corresponding to the molecular size of MED1 while in differentiation cocktail treated samples the intensity was increased considerably (Fig. 2.5E).

MED1 interacts with ESR1 and regulates target gene expression in HESC

MED1 subunit has the LXXLL signature motif which is important for interactions with nuclear receptors and has been shown to physically interact with several nuclear receptors, including thyroid receptor, vitamin D receptor, ESR1 and ESR2 [42-45]. In order to assess the possible interaction between MED1 and ESR1 in differentiating HESC, we performed co-

immunoprecipitation experiments. Immunoprecipitation was carried out on whole-cell extracts of differentiating HESC transduced with adenovirus expressing flag tagged ESR1 using anti-MED1 antibody. We detected ESR1 in the IP performed with the MED1 antibody (Fig. 2.6A), suggesting that MED1 and ESR1 interact in differentiating HESC. In parallel experiments, similarly prepared whole-cell extracts were immunoprecipitated with anti-flag M2 antibody coupled to beads, which recognize the exogenously expressed flag-tagged ESR1. As shown in Fig. 2.6B, MED1 co-precipitated with flag-ESR1 and the amount of MED1 that interacts with ESR1 was increased modestly in the presence of the differentiation cocktail compared to untreated sample. This increase was more prominent when MED1 was immunoprecipitated and the levels of co-precipitated ESR1 were monitored in E+P versus E+P+cAMP treated samples (Fig 2.6C), which coincided with the expression data of ESR1 target genes in the presence of E+P+cAMP compared to E+P.

MED1 is required for HESC differentiation:

As we documented that ESR1 was required for stromal differentiation and MED1 was a coactivator of ESR1 and a target of cAMP signaling, we investigated whether MED1 had a functional role during *in vitro* differentiation of HESCs. The silencing of MED1 expression resulted in a clear impairment in expression of the decidualization markers (Fig 2.7A) and a lack of morphological changes that associated with the differentiation process (Fig. 2.7B) suggesting that MED1 was required during *in vitro* differentiation of HESC. These data indicated that MED1 was a critical mediator of cAMP regulation of HESC decidualization and controlled the expression of a subset of the downstream targets of ESR1 including *WNT4* during the differentiation process (Fig. 2.7C). To test whether ESR1 brought MED1 to a target genomic region, we performed chromatin immunoprecipitation (ChIP) experiments on potential estrogen receptor binding sites in the regulatory regions of *WNT4* gene (Fig. 2.7D). These potential sites were recruited from available literature on ESR1 cistrome [46]. We found that MED1 was also recruited to these potential binding sites in the presence of cAMP+E+P while the recruitment of MED1 was minimal in the absence of cAMP when the cells were treated with E+P (Fig. 2.7E).

DISCUSSION

In the present study, we used primary cultures of HESC to establish the requirement of ESR1 for decidualization in human. We combined an ESR1 knock-down approach with gene expression profiling analysis to identify ESR1-dependent gene pathways in differentiating HESC. Our microarray analysis revealed that, in differentiating HESC, ESR1 controls several pathways belonging to various biological categories. Prominent among those are genes that encode cell cycle regulatory molecules. Specifically, genes involved in cell cycle progression, including *CCNE2*, *CCNA2*, *CDC20*, *CDK1*, *CDK2*, *NEK1*, *NEK2*, *SKP2* and *PLK4* were up-regulated in ESR1 depleted HESC. In contrast, genes involved in inhibition of the cell cycle, such as *CDKN1C* and *GAS1*, were down regulated when ESR1 was silenced. In agreement with these findings, we showed that stromal cell proliferation, as measured by BrdU incorporation, was increased in the absence of ESR1. It is remarkable that several of the cell cycle regulators found downstream of ESR1 in this present study were previously documented to be differentially expressed in secretory phase endometrium when compared to proliferative phase; as mRNA levels of *CCNA2*, *CCNE2*, *CDC20* were reported to be decreased and mRNA levels of *CDKN1C* and *GAS1* were found to be increased significantly in the secretory phase [47, 48]. However, to our knowledge, there had been no reports suggesting that these genes were downstream of ESR1. Wu et. al., using an immortalized endometrial cell line, studied the effects of various steroid receptor inhibitors on endometrial proliferation and showed that ICI had no inhibitory effect; in fact, at low levels it had a positive effect on cell proliferation [49] and our studies showed, for the first time, that ESR1 suppressed endometrial stromal cell proliferation and promoted their differentiation. These findings may be important in understanding the endometrial proliferation, and also should be considered when planning treatments for endometrial diseases such as endometriosis. Although estrogens generally induce proliferation of a variety of cells, this is one of the unique cases where estrogen receptor alpha suppresses cell cycle progression. The difference in the proliferative response may depend on the cell context, the expression levels of cofactors, the activities of other signaling pathways, epigenetic differences etc. For instance, estrogen treatment induces cell proliferation in MCF7 cells while represses proliferation of MDA-MB-231 cells. Previous study by Stender et al. showed that the difference in the proliferative response to estrogen in these two breast cancer cell lines correlated with the regulation of *E2F1* by estrogen receptor [50]. Additionally, Frietze et al. documented the

recruitment of ESR1 to the E2F1 promoter in MCF7 cells [51], suggesting that E2F1 may be a direct target of ESR1 and, depending on the cell context, its expression might be induced or repressed by ESR1. It is of interest to note that the E2F family members, which are key regulators of G1-S transition, also regulate the expression of *CCND3*, *CDC20* and *CCNA2* [50, 52]. This raised the interesting possibility that the ESR1 regulation of these cyclins and the cyclin-dependent kinase may be a direct consequence of ESR1 regulation of the E2Fs. Future chromatin immunoprecipitation experiments testing whether ESR1 directly regulates E2F1 or E2F2 are necessary to test this hypothesis.

Additionally, this study supports a central role for ESR1 in regulating HESC differentiation. *WNT4*, *FOXO1* and *PGR*, which are well known regulators of HESC differentiation, were induced downstream of ESR1. While the expressions of *FOXO1* and *WNT4* were drastically reduced within 24 h of the differentiation reaction in the absence of ESR1, the level of *PGR* was down-regulated significantly only at later time points such as 48h and 72 h. Therefore, it is possible that *FOXO1* and *WNT4* are *PGR* independent downstream targets of ESR1. *FOXO1*, a member of Forkhead family of transcription factors, is involved in cell cycle inhibition, defense against oxidative stress, DNA repair and apoptosis [53, 54] and importantly, in HESC decidualization; as Takano et al. reported, HESC differentiation was impaired in *FOXO1*-depleted cells [35]. Interestingly, they showed that *FOXO1* silencing promoted proliferation of HESC under differentiation conditions and several of the cell cycle-related genes reported to be regulated by *FOXO1* such as *CDKN1C*, *BUB1*, *NEK2* and *PLK4*, were also found to be downstream of ESR1 in our gene expression studies. This raises the possibility that some of the inhibitory effects of ESR1 on cell cycle progression of HESC could be mediated by *FOXO1*.

We observed that the expressions of ESR1 targets that we identified in this study were dependent on the presence of cAMP, which suggested a link between cAMP signaling and ESR1 signaling. We hypothesized that cAMP might be acting through PKA to phosphorylate the estrogen receptor or some other molecules such as kinases or cofactors that could modulate the receptor activity. We first tested whether ESR1 was phosphorylated by PKA and failed to detect any phosphorylation using PKA substrates antibody (data not shown). However, it is possible that this antibody does not recognize all targets of PKA. Therefore it is necessary to use phospho-ESR1 specific antibodies to test this possibility. Additionally, the effect of PKA on the

receptor might be indirect; the receptor might be modified post-translationally by a PKA target. One candidate signaling molecule was MAPK, which is known to modulate ESR1 activity through direct phosphorylation of the receptor on Ser118 residue. Our preliminary analysis showed that the Ser118 phosphorylation of ESR1 was primarily ligand, estrogen, dependent; and addition of cAMP increased Ser118 phosphorylation modestly (data not shown). Further detailed analyses are necessary to have a better understanding of this possibility.

Since there are several possible mechanisms through which cAMP signaling might modulate ESR1 activity, we decided to use an unbiased proteomic approach to identify the molecules which were getting phosphorylated by PKA during HESC differentiation. The potential targets that were detected by our preliminary experiments using mass spectrometry included MED1 and several cytoskeletal proteins. Among them, vimentin was previously shown to be phosphorylated by PKA. Phosphorylated vimentin showed decelerated filament formation *in vivo* [55]. Also, PKA-mediated phosphorylation of myofibrillar proteins was reported to alter the mechanical properties of myocardium [56]. We believe that phosphorylation of cytoskeletal elements by PKA may be important for the differentiation process as decidualization involves major cytoskeletal remodeling. Amongst the potential targets of PKA, we focused on the cofactor MED1, a subunit of the Mediator complex, which interacts with a variety of nuclear receptors and forms a bridge with the general transcription machinery [40, 42-45]. MED1 is a phospho-protein and in support of our finding, it was previously identified as an *in vitro* substrate for PKA [41]. We showed that MED1 and ESR1 interacted in HESC and with addition of cAMP+E+P, both were recruited to ESR1 binding sites previously identified in WNT4 regulatory regions [46]. Previous work indicated that MAPK phosphorylation of MED1 stimulated transcriptional coactivation activity of MED1 [57]. Belakavadi et al. showed that while phosphorylation of MED1 by ERK did not alter its interaction with the thyroid receptor (TR), it promoted its association with the Mediator complex and enhanced TR dependent transcription [58]. We speculate that the phosphorylation of MED1 by PKA may present a mechanism by which cAMP effects could be linked to gene transcription as we documented that the interaction between ESR1 and MED1 was increased with the addition of cAMP to E+P treatment. A similar mechanism between ESR1 and coactivator-associated arginine methyltransferase 1 (CARM1) was identified by Carascossa et al. in MCF7 cells; as they showed that phosphorylation of CARM1 by PKA was necessary and sufficient for its direct binding to

the estrogen receptor while phosphorylation of the estrogen receptor by PKA was not required for this interaction [59]. Therefore, further characterization of the possible functional roles of the PKA phosphorylation of MED1 by examining its interactions with the Mediator complex upon phosphorylation and consequent effects on transcriptional activity of ESR1 is necessary. It should also be considered that MED1 serves as a cofactor of several other nuclear receptors, including RAR and PPARG. Interestingly, these molecules were reported to be negative regulators of HESC differentiation and their transcriptional activity might also be affected by post translational modification of MED1 which might have important regulatory roles in progression of HESC differentiation [60, 61].

Finally, we showed that the HESC differentiation was severely impaired in MED1 depleted cells. Interestingly, we did not observe impairment in their proliferation (data not shown). A similar role of MED1 in adipogenesis was reported by Ge et al. using fibroblast cells lacking MED1. These cells were reported to be impaired in PPARG stimulated adipogenesis while they could differentiate into myocytes through MyoD stimulated myogenesis [62].

In summary, our study revealed that ESR1 had two main roles during human decidualization. First, it suppressed the proliferation of HESC. We hypothesize that ESR1 may inhibit proliferation of stromal cells by suppressing the expression of E2F1 and E2F2 and their downstream cell cycle regulatory factors and also through upregulation of FOXO1 as this molecule was previously shown to be a suppressor of HESC proliferation during differentiation [35]. Second, ESR1 regulated the differentiation of HESC by inducing the expression of important regulators of the differentiation process, namely *FOXO1*, *WNT4* and *PGR*. Future studies will address the precise mechanisms by which ESR1 conducts these regulatory events. Additionally, we identified MED1 as a potential substrate of PKA and showed that it interacted with ESR1. Moreover, we showed that differentiation was severely impaired in MED1 depleted cells, suggesting that MED1, upon phosphorylation by PKA, was indeed required for differentiation of HESC and expression of ESR1 downstream genes which might be one of the signaling mechanisms cAMP employed to drive stromal differentiation together with the steroid hormones (Fig. 2.8).

REFERENCES

1. Wang, H. and S.K. Dey, *Roadmap to embryo implantation: clues from mouse models*. Nat Rev Genet, 2006. **7**(3): p. 185-99.
2. Carson, D.D., et al., *Embryo implantation*. Dev Biol, 2000. **223**(2): p. 217-37.
3. Ramathal, C.Y., et al., *Endometrial decidualization: of mice and men*. Semin Reprod Med, 2010. **28**(1): p. 17-26.
4. Brosens, J.J., et al., *A role for menstruation in preconditioning the uterus for successful pregnancy*. Am J Obstet Gynecol, 2009. **200**(6): p. 615 e1-6.
5. Brosens, J.J., R. Pijnenborg, and I.A. Brosens, *The myometrial junctional zone spiral arteries in normal and abnormal pregnancies: a review of the literature*. Am J Obstet Gynecol, 2002. **187**(5): p. 1416-23.
6. Salker, M., et al., *Natural selection of human embryos: impaired decidualization of endometrium disables embryo-maternal interactions and causes recurrent pregnancy loss*. PLoS One, 2010. **5**(4): p. e10287.
7. Gellersen, B., I.A. Brosens, and J.J. Brosens, *Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives*. Semin Reprod Med, 2007. **25**(6): p. 445-53.
8. Chang, E.C., et al., *Estrogen Receptors alpha and beta as determinants of gene expression: influence of ligand, dose, and chromatin binding*. Mol Endocrinol, 2008. **22**(5): p. 1032-43.
9. Hewitt, S.C., J.C. Harrell, and K.S. Korach, *Lessons in estrogen biology from knockout and transgenic animals*. Annu Rev Physiol, 2005. **67**: p. 285-308.
10. Xu, J., et al., *Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene*. Science, 1998. **279**(5358): p. 1922-5.

11. Gellersen, B. and J. Brosens, *Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair*. J Endocrinol, 2003. **178**(3): p. 357-72.
12. Klemmt, P.A.B., et al., *Stromal cells from endometriotic lesions and endometrium from women with endometriosis have reduced decidualization capacity*. Fertil Steril, 2006. **85**(3): p. 564-572.
13. Tang, B., S. Guller, and E. Gurpide, *Mechanisms involved in the decidualization of human endometrial stromal cells*. Acta Eur Fertil, 1993. **24**(5): p. 221-3.
14. Irwin, J.C., et al., *Hormonal regulation of human endometrial stromal cells in culture: an in vitro model for decidualization*. Fertil Steril, 1989. **52**(5): p. 761-8.
15. Buzzio, O.L., et al., *FOXO1A differentially regulates genes of decidualization*. Endocrinology, 2006. **147**(8): p. 3870-6.
16. Aghajanova, L., et al., *Steroidogenic enzyme and key decidualization marker dysregulation in endometrial stromal cells from women with versus without endometriosis*. Biol Reprod, 2009. **80**(1): p. 105-14.
17. Telgmann, R., et al., *Activated protein kinase A is required for differentiation-dependent transcription of the decidual prolactin gene in human endometrial stromal cells*. Endocrinology, 1997. **138**(3): p. 929-937.
18. Jones, M.C., et al., *Regulation of the SUMO pathway sensitizes differentiating human endometrial stromal cells to progesterone*. Proc Natl Acad Sci U S A, 2006. **103**(44): p. 16272-7.
19. Brosens, J.J., N. Hayashi, and J.O. White, *Progesterone receptor regulates decidual prolactin expression in differentiating human endometrial stromal cells*. Endocrinology, 1999. **140**(10): p. 4809-20.
20. Pansini, F., et al., *Sex steroid hormones influence the cAMP content in human endometrium during the menstrual cycle*. Gynecol Obstet Invest, 1984. **18**(4): p. 174-7.

21. Maruyama, T. and Y. Yoshimura, *Molecular and cellular mechanisms for differentiation and regeneration of the uterine endometrium*. Endocr J, 2008. **55**(5): p. 795-810.
22. Skalhogg, B.S. and K. Tasken, *Specificity in the cAMP/PKA signaling pathway. Differential expression, regulation, and subcellular localization of subunits of PKA*. Front Biosci, 2000. **5**: p. D678-93.
23. Fimia, G.M. and P. Sassone-Corsi, *Cyclic AMP signalling*. J Cell Sci, 2001. **114**(Pt 11): p. 1971-2.
24. Howe, A.K., *Regulation of actin-based cell migration by cAMP/PKA*. Biochim Biophys Acta, 2004. **1692**(2-3): p. 159-74.
25. Bostock, C.J., D.M. Prescott, and J.B. Kirkpatrick, *An evaluation of the double thymidine block for synchronizing mammalian cells at the G1-S border*. Exp Cell Res, 1971. **68**(1): p. 163-8.
26. ST, A.G.; Available from: http://www.affymetrix.com/estore/browse/products.jsp?navMode=34000&productId=131453&navAction=jump&aId=productsNav#1_1.
27. analysis, I.p.; Available from: <http://www.ingenuity.com/>.
28. Pearce, L.R., D. Komander, and D.R. Alessi, *The nuts and bolts of AGC protein kinases*. Nat Rev Mol Cell Biol, 2010. **11**(1): p. 9-22.
29. Signaling, C.; Available from: <http://www.cellsignal.com/pdf/9624.pdf>.
30. Mylonas, I., et al., *Immunohistochemical analysis of estrogen receptor alpha, estrogen receptor beta and progesterone receptor in normal human endometrium*. Acta Histochem, 2004. **106**(3): p. 245-52.
31. Lannigan, D.A., *Estrogen receptor phosphorylation*. Steroids, 2003. **68**(1): p. 1-9.
32. Miller, J.P., et al., *Interweaving the cell cycle machinery with cell differentiation*. Cell Cycle, 2007. **6**(23): p. 2932-8.

33. Li, Q., et al., *Bone morphogenetic protein 2 functions via a conserved signaling pathway involving Wnt4 to regulate uterine decidualization in the mouse and the human*. J Biol Chem, 2007. **282**(43): p. 31725-32.
34. Franco, H.L., et al., *WNT4 is a key regulator of normal postnatal uterine development and progesterone signaling during embryo implantation and decidualization in the mouse*. FASEB J, 2011. **25**(4): p. 1176-87.
35. Takano, M., et al., *Transcriptional cross talk between the forkhead transcription factor forkhead box O1A and the progesterone receptor coordinates cell cycle regulation and differentiation in human endometrial stromal cells*. Mol Endocrinol, 2007. **21**(10): p. 2334-49.
36. ; Available from: http://www.biolog.de/uploads/tx_eshop/B%20007.pdf
37. Enserink, J.M., et al., *A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK*. Nat Cell Biol, 2002. **4**(11): p. 901-6.
38. Brar, A.K., et al., *Progesterone-dependent decidualization of the human endometrium is mediated by cAMP*. Endocrine, 1997. **6**(3): p. 301-307.
39. Davies, S.P., et al., *Specificity and mechanism of action of some commonly used protein kinase inhibitors*. Biochemical Journal, 2000. **351**: p. 95-105.
40. Malik, S., et al., *Structural and functional organization of TRAP220, the TRAP/mediator subunit that is targeted by nuclear receptors*. Mol Cell Biol, 2004. **24**(18): p. 8244-54.
41. Misra, P., et al., *Phosphorylation of transcriptional coactivator peroxisome proliferator-activated receptor (PPAR)-binding protein (PBP). Stimulation of transcriptional regulation by mitogen-activated protein kinase*. J Biol Chem, 2002. **277**(50): p. 48745-54.
42. Yuan, C.X., et al., *The TRAP220 component of a thyroid hormone receptor- associated protein (TRAP) coactivator complex interacts directly with nuclear receptors in a ligand-dependent fashion*. Proc Natl Acad Sci U S A, 1998. **95**(14): p. 7939-44.

43. Zhang, X., et al., *MED1/TRAP220 exists predominantly in a TRAP/ Mediator subpopulation enriched in RNA polymerase II and is required for ER-mediated transcription*. Mol Cell, 2005. **19**(1): p. 89-100.
44. Warnmark, A., et al., *Differential recruitment of the mammalian mediator subunit TRAP220 by estrogen receptors ERalpha and ERbeta*. J Biol Chem, 2001. **276**(26): p. 23397-404.
45. Kang, Y.K., et al., *The TRAP/Mediator coactivator complex interacts directly with estrogen receptors alpha and beta through the TRAP220 subunit and directly enhances estrogen receptor function in vitro*. Proc Natl Acad Sci U S A, 2002. **99**(5): p. 2642-7.
46. Carroll, J.S., et al., *Genome-wide analysis of estrogen receptor binding sites*. Nat Genet, 2006. **38**(11): p. 1289-97.
47. Talbi, S., et al., *Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women*. Endocrinology, 2006. **147**(3): p. 1097-121.
48. Groothuis, P.G., et al., *Estrogen and the endometrium: lessons learned from gene expression profiling in rodents and human*. Hum Reprod Update, 2007. **13**(4): p. 405-17.
49. Wu, Y. and S.W. Guo, *Inhibition of proliferation of endometrial stromal cells by trichostatin A, RU486, CDB-2914, N-acetylcysteine, and ICI 182780*. Gynecol Obstet Invest, 2006. **62**(4): p. 193-205.
50. Stender, J.D., et al., *Estrogen-regulated gene networks in human breast cancer cells: involvement of E2F1 in the regulation of cell proliferation*. Mol Endocrinol, 2007. **21**(9): p. 2112-23.
51. Frietze, S., et al., *CARM1 regulates estrogen-stimulated breast cancer growth through up-regulation of E2F1*. Cancer Res, 2008. **68**(1): p. 301-6.
52. Ma, Y., et al., *Regulation of the cyclin D3 promoter by E2F1*. J Biol Chem, 2003. **278**(19): p. 16770-6.

53. Kops, G.J., et al., *Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors*. Mol Cell Biol, 2002. **22**(7): p. 2025-36.
54. Kajihara, T., et al., *Differential expression of FOXO1 and FOXO3a confers resistance to oxidative cell death upon endometrial decidualization*. Mol Endocrinol, 2006. **20**(10): p. 2444-55.
55. Eriksson, J.E., et al., *Specific in vivo phosphorylation sites determine the assembly dynamics of vimentin intermediate filaments*. J Cell Sci, 2004. **117**(Pt 6): p. 919-32.
56. Patel, J.R., et al., *PKA accelerates rate of force development in murine skinned myocardium expressing alpha- or beta-tropomyosin*. Am J Physiol Heart Circ Physiol, 2001. **280**(6): p. H2732-9.
57. Pandey, P.K., et al., *Activation of TRAP/mediator subunit TRAP220/Med1 is regulated by mitogen-activated protein kinase-dependent phosphorylation*. Mol Cell Biol, 2005. **25**(24): p. 10695-710.
58. Belakavadi, M., et al., *MED1 phosphorylation promotes its association with mediator: implications for nuclear receptor signaling*. Mol Cell Biol, 2008. **28**(12): p. 3932-42.
59. Carascossa, S., et al., *CARM1 mediates the ligand-independent and tamoxifen-resistant activation of the estrogen receptor alpha by cAMP*. Genes Dev, 2010. **24**(7): p. 708-19.
60. Brar, A.K., et al., *Retinoic acid suppresses in-vitro decidualization of human endometrial stromal cells*. Mol Hum Reprod, 1996. **2**(3): p. 185-93.
61. Chang, H.J., et al., *Transforming growth factor (TGF)-beta1-induced human endometrial stromal cell decidualization through extracellular signal-regulated kinase and Smad activation in vitro: peroxisome proliferator-activated receptor gamma acts as a negative regulator of TGF-beta1*. Fertil Steril, 2008. **90**(4 Suppl): p. 1357-65.
62. Ge, K., et al., *Transcription coactivator TRAP220 is required for PPAR gamma 2-stimulated adipogenesis*. Nature, 2002. **417**(6888): p. 563-7.

FIGURES AND TABLES

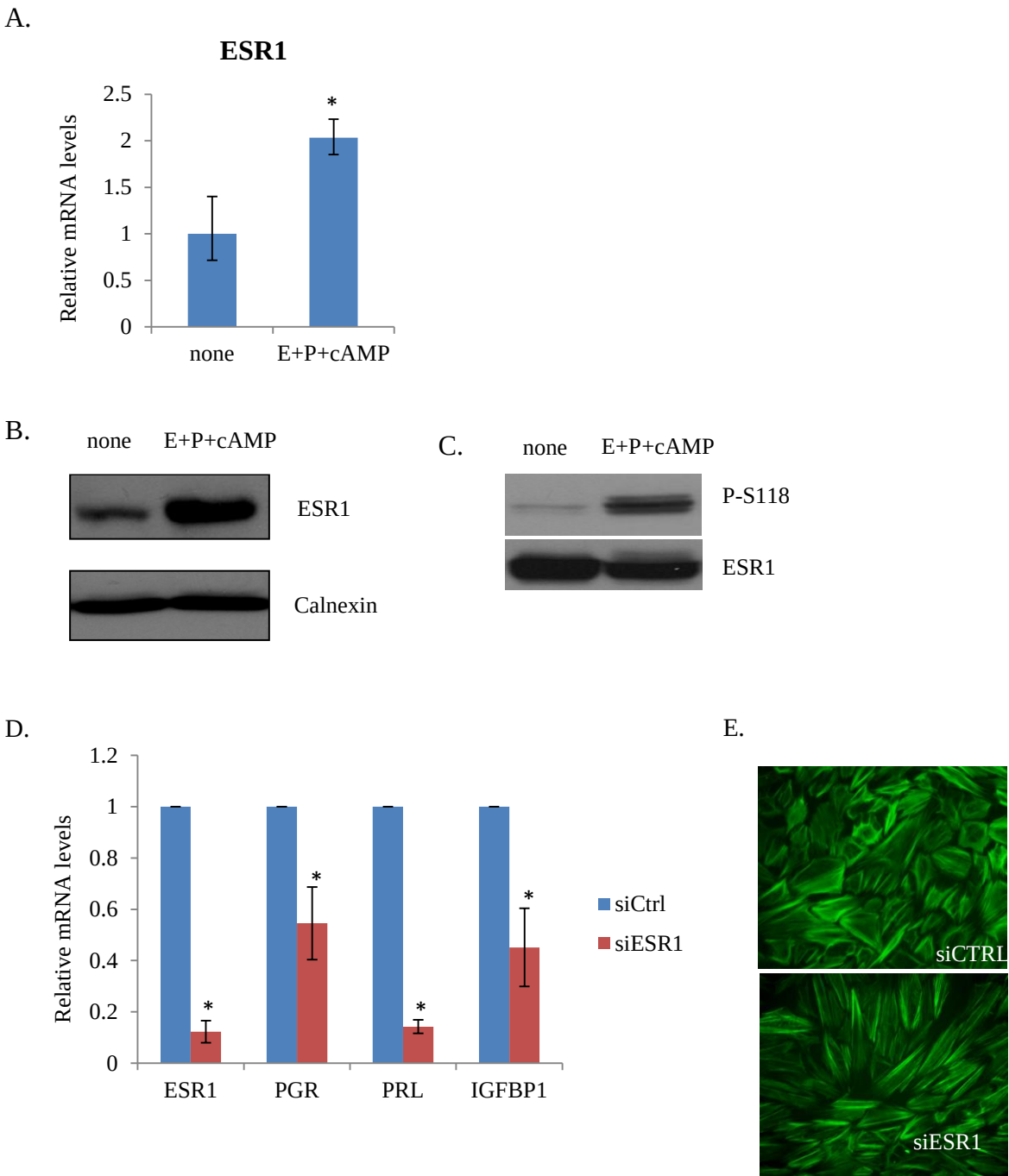


Figure 2.1: ESR1 is required for HESC differentiation

Figure 2.1 cont.: **A.** HES C were either untreated or treated with differentiation cocktail. Cells were harvested at 24h following treatment. Total RNA was isolated and cDNAs were prepared. cDNAs were subjected to real-time PCR using gene specific primers for 36B4 and ESR1. PCR data are normalized to 36B4 mRNA levels. Relative fold changes were normalized with respect to the untreated sample. Error bars represent $\pm$ standard deviation of at least three independent experiments. **B.** HESC were either untreated or treated with differentiation cocktail as indicated on the figure. Cells were lysed at 24h following treatment, analyzed by SDS-PAGE and subjected to Western Blotting using antibodies directed against ESR1 and Calnexin. Calnexin immunoblotting serves as a loading control. **C.** HESC were transfected with adenovirus expressing flag tagged wild type ESR1 in charcoal stripped serum containing medium for 24h. After viral transfection, medium was removed and HESC were either untreated or treated with differentiation cocktail for 3h. Cells were harvested and whole cell protein extracts were prepared. Equal amounts of protein of each sample were analyzed by SDS-PAGE. Western blot was performed using either anti-ESR1 phosphoserine (α -pS118) or anti-ESR1 antibodies. Figure is the representative of three independent experiments. **D.** HESCs were transfected with siRNA targeting ESR1 or scrambled siRNA (control). 48h after transfection, HESCs were treated with differentiation cocktail to initiate *in vitro* differentiation. Cells were harvested at 72h following differentiation cocktail treatment. Total RNA was isolated and cDNAs were prepared. cDNAs were subjected to real-time PCR using gene specific primers for ESR1, PRL, IGFBP1 and WNT4. PCR data are normalized to 36B4 mRNA levels. Relative fold changes were normalized with respect to control non-targeting siRNA treatment. Figure is the representative of three independent experiments. **E.** HESCs were seeded on 4-well chamber slides and cells were transfected with siRNA targeting ESR1 or scrambled siRNA (control). 48h after transfection, HESCs were treated with differentiation cocktail to initiate *in vitro* differentiation. At 96h of differentiation, cells were fixed and stained as explained in materials and methods. Top panel is a reference representing the typical morphological transformation of HESC into differentiated HESC. Phalloidin (green) staining of HESCs are shown.

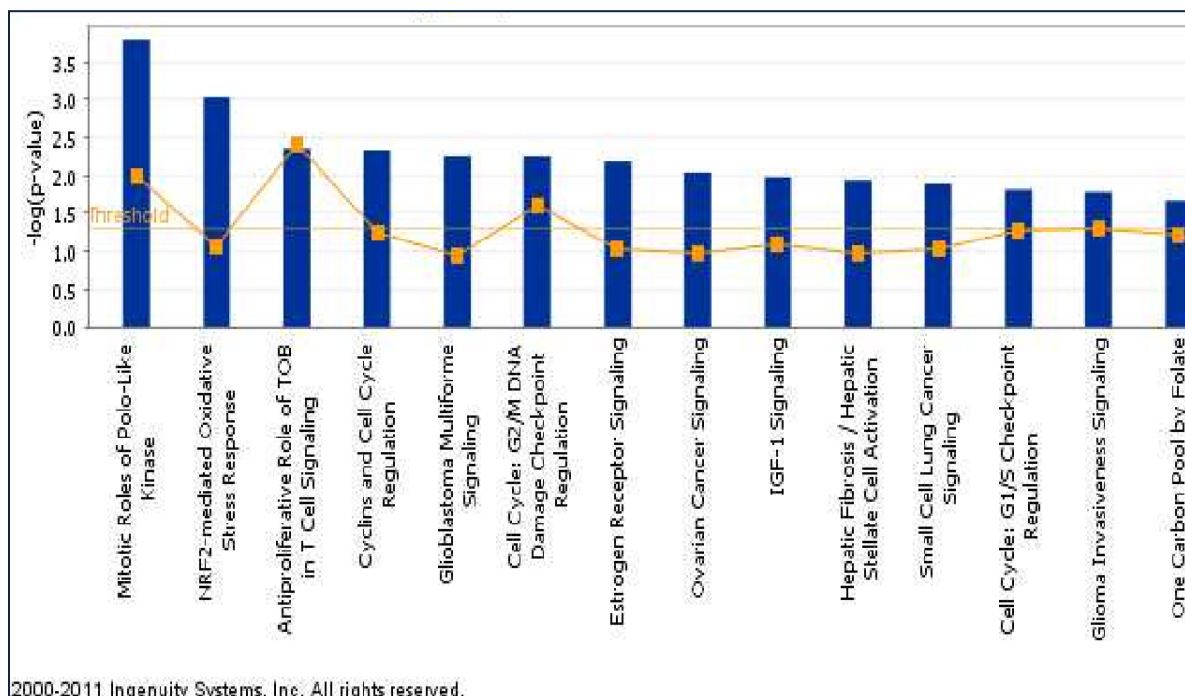


Table 2.1: Ingenuity Pathway Analysis of Canonical Pathways for the ESR1 dependent Genes

Genes with altered expression in ESR1 siRNA transfected cells compared to control siRNA transfected cells were categorized by canonical pathways using Ingenuity pathway analysis software (IPA).

Symbol	Entrez Gene Name	fold change (mean)
AURKA	Aurora kinase A	1.6
AURKB	Aurora kinase B	1.8
BUB1	Budding Uninhibited By Benzimidazoles 1	1.8
BUB1B	budding uninhibited by benzimidazoles 1 homolog beta (yeast)	2.0
CCNA2	cyclin A2	1.8
CCND3	cyclin D3	1.3
CCNE2	cyclin E2	1.7
CDC20	cell division cycle 20 homolog (S. cerevisiae)	1.8
CDK1	cyclin-dependent kinase 1	1.9
CDK2	cyclin-dependent kinase 2	1.3
CKS2	CDC28 protein kinase regulatory subunit 2	1.5
E2F8	E2F transcription factor 8	1.3
ESPL1	extra spindle pole bodies homolog 1 (S. cerevisiae)	1.4
HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	1.3
KIF23	kinesin family member 23	1.5
NEK2	NIMA (never in mitosis gene a)-related kinase 2	1.8
NUSAP1	nucleolar and spindle associated protein 1	1.8
PLK4	polo-like kinase 4	1.9
SKP2	S-phase kinase-associated protein 2 (p45)	1.4
SLK	STE20-like kinase	1.5
TFDP1	transcription factor Dp-1	1.2

Table 2.2: List of cell cycle related molecules downstream of ESR1 during HESC differentiation

List of cell cycle related genes identified in our microarray analysis with increased expression in siESR1 transfected samples compared to control siRNA transfected samples.

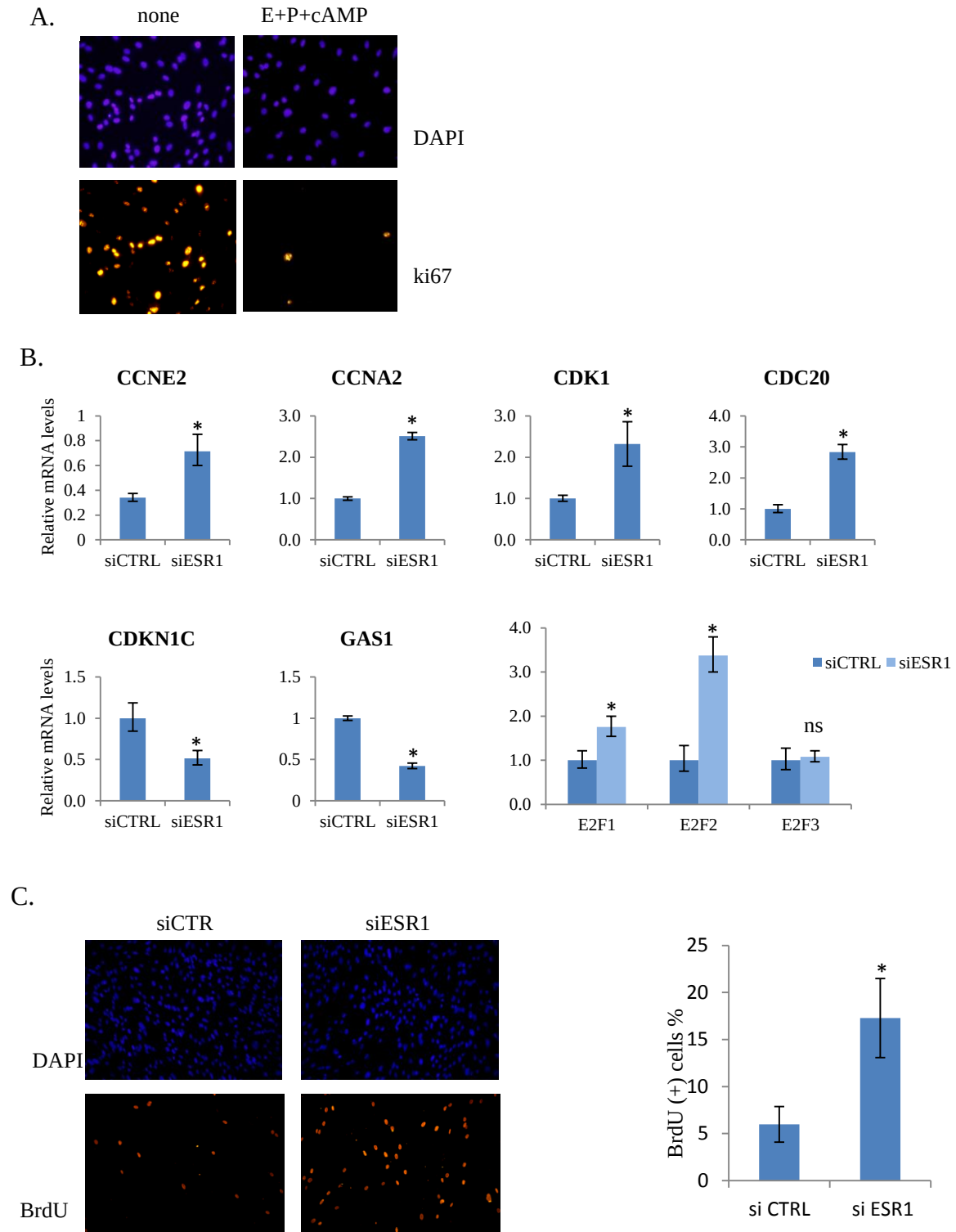


Figure 2.2: ESR1 suppresses the proliferation of HESC during *in vitro* differentiation

Figure 2.2 cont.:

- A. Cell cycle progression is significantly reduced during *in vitro* differentiation. HESCs were seeded on 4-well chamber slides and cells were either untreated or treated with E+P+cAMP for 72h. Cells were fixed and subjected to immunofluorescence, using an antibody specific for Ki67. Positive staining for Ki67 is indicated in red and DAPI stained nuclei are shown in blue.
- B. HESCs were transfected with siRNA targeting ESR1 or scrambled siRNA (control). 48h after transfection, HESCs were treated with differentiation cocktail to initiate *in vitro* differentiation. Cells were harvested at 24h following differentiation cocktail treatment. Total RNA was isolated and cDNAs were prepared. cDNAs were subjected to real-time PCR using gene specific primers for *36B4*, *ESR1*, *CCND3*, *CCNA2*, *CDC20*, *GAS1*, *CDKN1C*, *E2F1*, *E2F2* and *E2F3*. PCR data are normalized to *36B4* mRNA levels. Relative fold changes were normalized with respect to control non-targeting siRNA treatment. Error bars represent $\pm$ standard deviation of at least three independent experiments.
- C. HESC were synchronized at G1/S stage by double thymidine block and were transfected with siRNA targeting ESR1 or non-targeting control siRNA. 24h after transfection, HESCs were treated with differentiation cocktail to initiate *in vitro* differentiation. At 18h following differentiation cocktail treatment, BrdU was added to the medium (100 μ M). At 6h following BrdU addition (24h of differentiation), cells were fixed and subjected to immunofluorescence to detect BrdU positive cells, which are indicated in red. DAPI stained nuclei are shown in blue. Images are the representatives of three independent experiments. The graph shows the mean values of percentages of BrdU positive cells calculated from five different fields, error bars represent $\pm$ standard deviation.

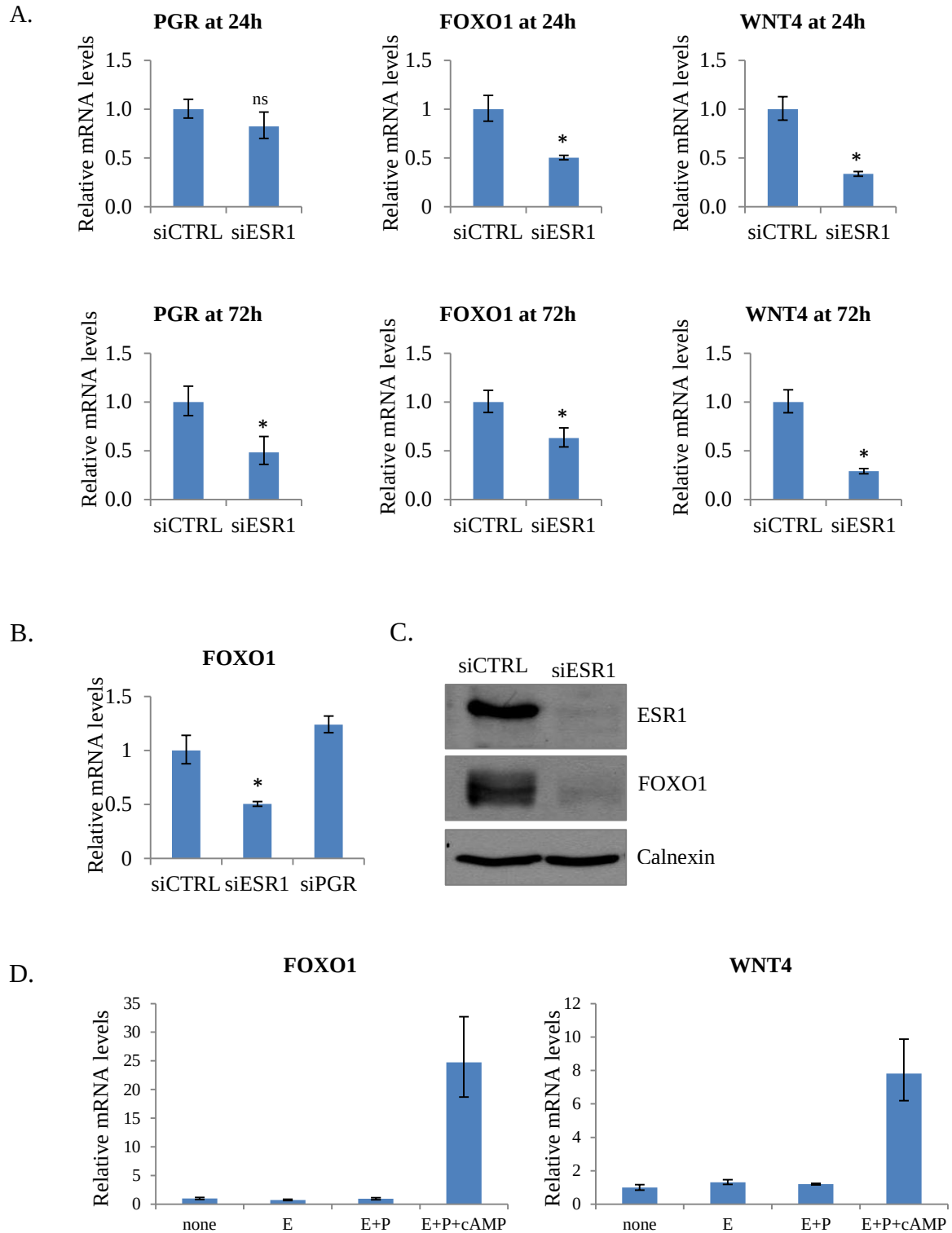


Figure 2.3: FOXO1, WNT4 and PGR are downstream of ESR1 during HESC differentiation.

Figure 2.3 cont.:

- A. HESCs were transfected with siRNA targeting ESR1 or scrambled siRNA (control). 48h after transfection, HESCs were treated with differentiation cocktail to initiate *in vitro* differentiation. Cells were harvested at 24h and 72h following differentiation cocktail treatment. Total RNA was isolated and cDNAs were prepared. cDNAs were subjected to real-time PCR using gene specific primers for 36B4, PGR, FOXO1 and WNT4. PCR data are normalized to 36B4 mRNA levels. Relative fold changes were normalized with respect to control non-targeting siRNA treatment. Error bars represent $\pm$ standard deviation.
- B. HESCs were transfected with siRNA targeting ESR1, or PGR or scrambled siRNA (control). 48h after transfection, HESCs were treated with differentiation cocktail to initiate *in vitro* differentiation. Cells were harvested at 24h following differentiation cocktail treatment. Total RNA was isolated and cDNAs were prepared. cDNAs were subjected to real-time PCR using gene specific primers for 36B4 and FOXO1. PCR data are normalized to 36B4 mRNA levels. Relative fold changes were normalized with respect to control non-targeting siRNA treatment. This is a representative of four independent experiments.
- C. HESCs were transfected with siRNA targeting ESR1, or PGR or scrambled siRNA (control). 48h after transfection, HESCs were treated with differentiation cocktail to initiate *in vitro* differentiation. Cells were lysed at 24h following treatment, analyzed by SDS-PAGE and subjected to western blotting using antibodies directed against ESR1, PGR, FOXO1 and Calnexin. Calnexin immunoblotting serves as a loading control.
- D. HESCs were hormone starved in 2% charcoal stripped serum containing medium for 48h. Then, cells were either untreated or treated with E+P or E+P+cAMP. Cells were harvested at 24h following each treatment. Total RNA was isolated and cDNAs were prepared. cDNAs were subjected to real-time PCR using gene specific primers for 36B4, FOXO1 and WNT4. PCR data are normalized to 36B4 mRNA levels. Relative fold changes were normalized with respect to untreated sample indicated as none in the figure.

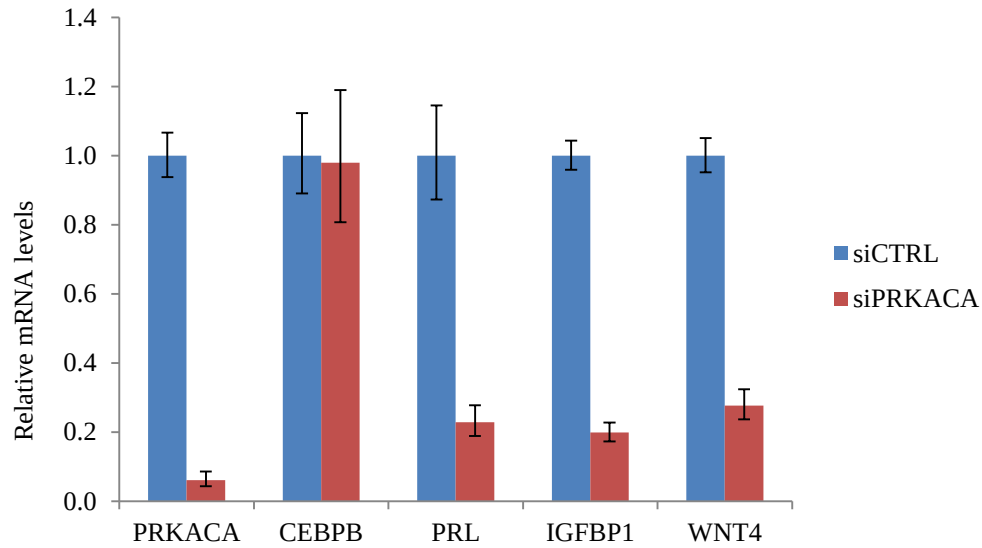


Figure 2.4: PKA signaling is required for cAMP induced differentiation of HESC

HESCs were transfected with siRNA targeting Protein Kinase A catalytic subunit alpha or scrambled siRNA (control). 48h after transfection, HESCs were treated with differentiation cocktail to initiate *in vitro* differentiation. Cells were harvested at 48h following differentiation cocktail treatment. Total RNA was isolated and cDNAs were prepared. cDNAs were subjected to real-time PCR using gene specific primers for PRKACA, PRL, IGFBP1 and WNT4. PCR data are normalized to 36B4 mRNA levels. Relative fold changes were normalized with respect to control non-targeting siRNA treatment. Error bars represent $\pm$ SD of three separate measurements.

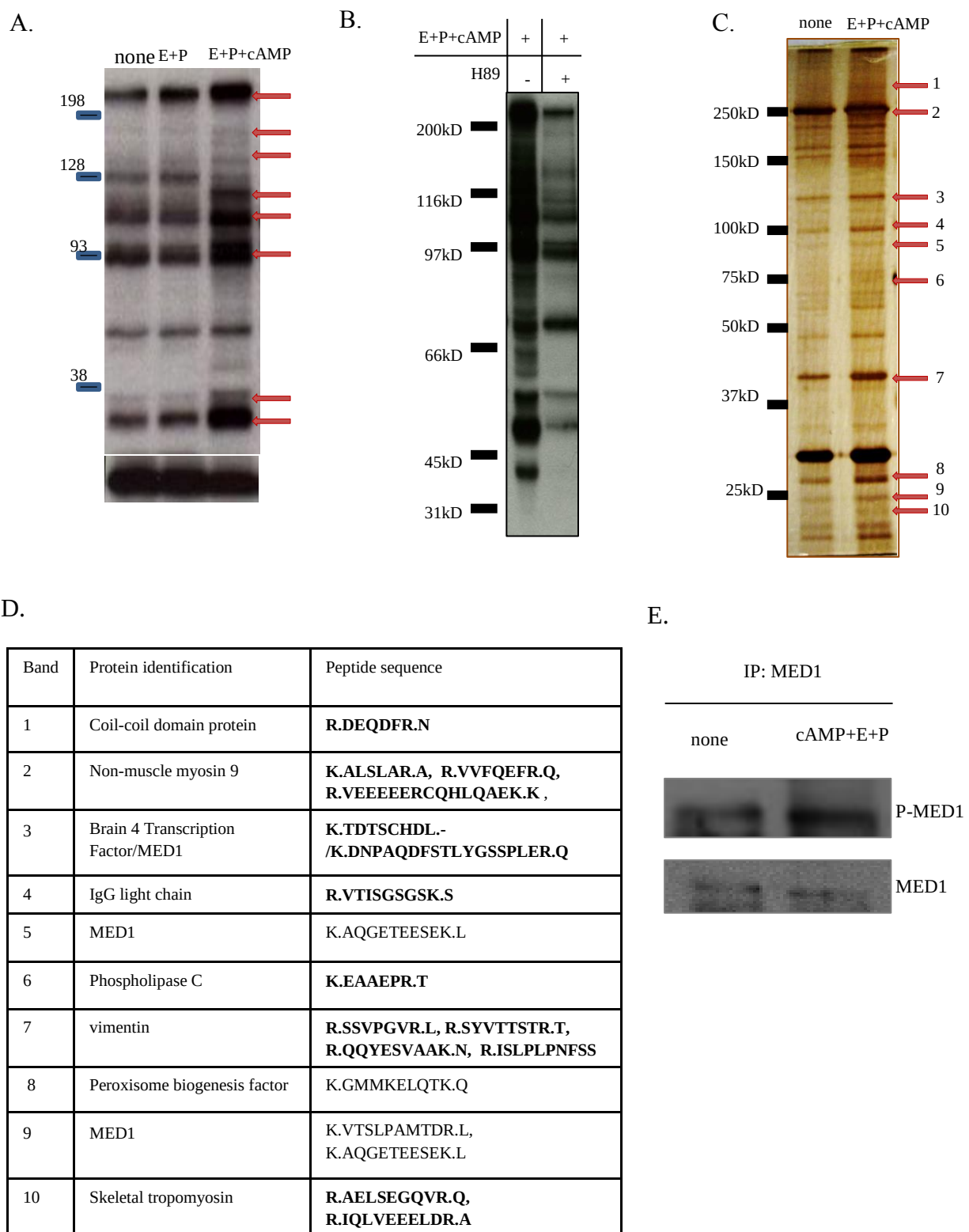


Figure 2.5: Identification of proteins phosphorylated by PKA during HESC differentiation

Figure 2.5 cont.:

- A. HESC were untreated or treated with E+P or E+P+cAMP. Cells were lysed at 3h following treatment, analyzed by SDS-PAGE and subjected to Western Blotting using phospho-PKA substrate antibody. Data is representative of six independent experiments.
- B. HESC were treated with either vehicle or with the PKA inhibitor H89. Following inhibitor treatment, cells were treated with differentiation cocktail, composed of E+P+cAMP. Cells were lysed at 24h following treatment, analyzed by SDS-PAGE and subjected to Western Blotting using phospho-PKA substrate antibody. Lane 1: vehicle, with differentiation cocktail, lane 2: H89, with differentiation cocktail.
- C. HESC were either untreated or treated with differentiation cocktail, composed of E+P+cAMP. At 24h following treatment, cells were harvested and lysed. Whole cell lysates from each sample were immunoprecipitated with phospho-PKA substrates antibody to pull down phosphorylated targets of PKA. Bound proteins were eluted and analyzed by SDS PAGE followed by silver staining as explained in materials and methods. Arrows indicate the bands which are submitted for protein identification by mass spectrometry.
- D. HESC were either untreated or treated with differentiation cocktail, composed of E+P+cAMP for 24h. Whole cell lysates from HESC were immunoprecipitated with MED1 antibody. The immunoprotein complexes were then probed by western blot using phospho-PKA substrate antibody or MED1 antibody as indicated in the figure.

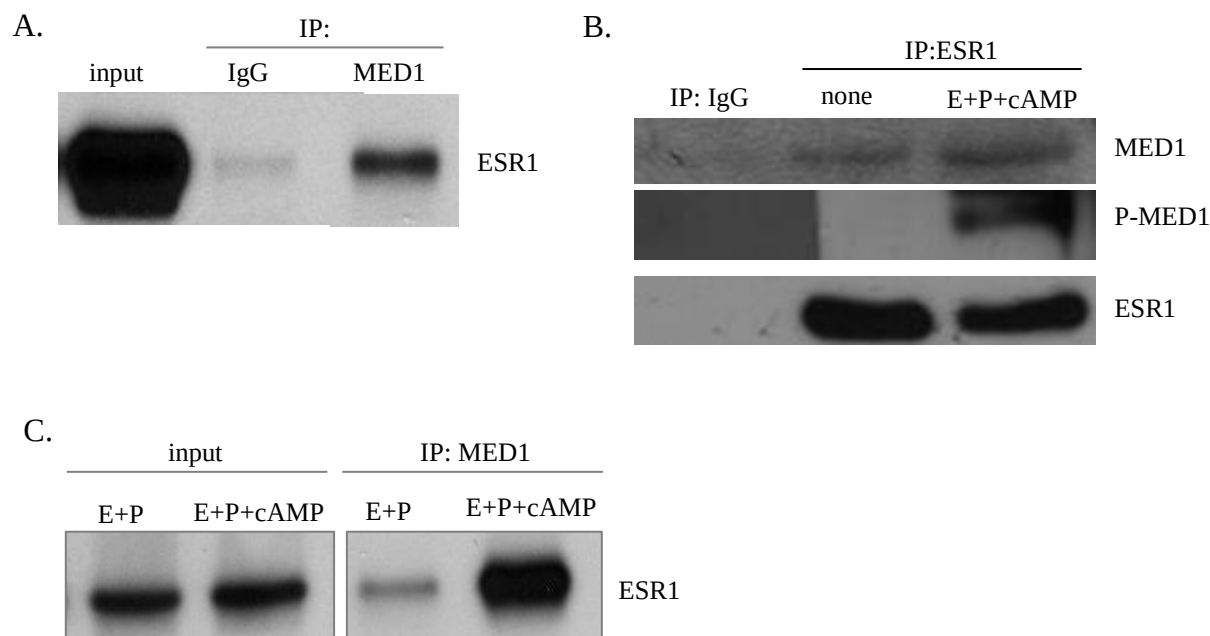


Figure 2.6: Interaction between MED1 and ESR1 in HESC

- A. HESC were infected with adenovirus expressing flag tagged ESR1 for 24h, following transfection, cells were treated with the differentiation cocktail composed of E+P+CAMP for 3h. Whole cell lysates from HESC were immunoprecipitated with MED1 antibody or normal rabbit IgG as a negative control. The immunoprotein complexes were then probed by western blot using ESR1 antibody.
- B. HESC were infected with adenovirus expressing flag tagged ESR1 for 24h, following transfection, cells were treated with the differentiation cocktail for 3h. Whole cell lysates from HESC were immunoprecipitated with anti-flag M2 antibody coupled to beads to pull down exogenously expressed flag-tagged ESR1. Bound proteins were eluted with FLAG peptide and analyzed by SDS PAGE and western blot using MED1 antibody, phospho-PKA substrate antibody and ESR1 antibody. Figure is the representative of two independent experiments.
- C. HESC were infected with adenovirus expressing flag tagged ESR1 for 24h, following transfection, cells were treated with E+P or E+P+cAMP for 3h. Whole cell lysates from HESC were immunoprecipitated with anti-MED1 antibody coupled to beads. Bound proteins were analyzed by SDS PAGE and western blot using ESR1 antibody. Figure is the representative of three independent experiments.

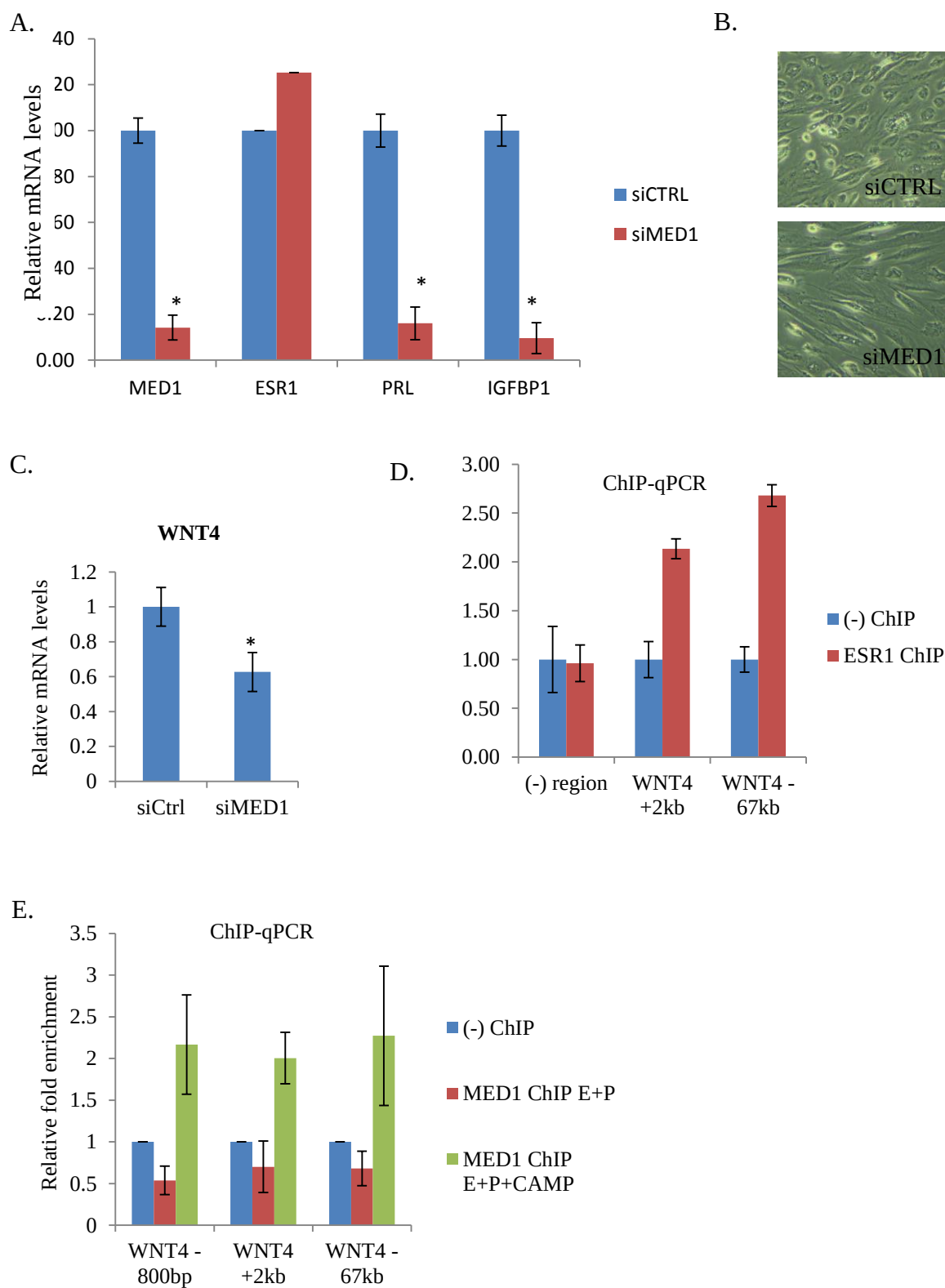


Figure 2.7: MED1 is required for cAMP induced differentiation of HESC.

Fig. 2.7 cont.:

- A. HESCs were transfected with siRNA targeting MED1 or non-targeting scrambled siRNA for 48h. After siRNA treatment, cells were treated with differentiation cocktail composed of 0.5mM 8-Br-cAMP + 10 μ M P + 10nM E for 72h. Real time-PCR was performed to analyze gene expression using specific primers for MED1, ESR1, PRL and IGFBP1; values are normalized to control siRNA treatment. The graph represents mean $\pm$ SD of three independent experiments.
- B. HESCs were transfected with siRNA targeting MED1 or control siRNA. 48h after transfection, HESCs were treated with differentiation cocktail to initiate *in vitro* differentiation. At 72h of differentiation, cells were visualized under bright field microscope to monitor decidual morphology. Data is representative of four experiments.
- C. HESCs were transfected with siRNA targeting MED1 or non-targeting scrambled siRNA for 48h. After siRNA treatment, cells were treated with differentiation cocktail. Cells were harvested at 24h following differentiation cocktail addition. For gene expression analysis, Q-PCR was performed using specific primers for WNT4; values are normalized to control siRNA treatment. The graph represents mean $\pm$ SD of six independent experiments.
- D. HESC were transduced with recombinant adenovirus harboring flag-tagged ESR1 for 24 h. Samples were treated with E+P+cAMP for 1h. ChIP was performed as described in material and methods section. Chromatin enrichment was quantified by real-time PCR using primers flanking potential ESR1 binding sites upstream of and in the *WNT4* gene. The experiment was repeated twice and representative data are shown.
- E. ChIP was performed under similar conditions with section D to immunoprecipitate MED1 bound chromatin as explained in materials and methods. Primers flanking potential ESR1 bound regulatory regions and promoter of *WNT4* gene, indicated as WNT4 -700bp, were used to quantify chromatin enrichment. The graph represents mean $\pm$ SD of three independent experiments.

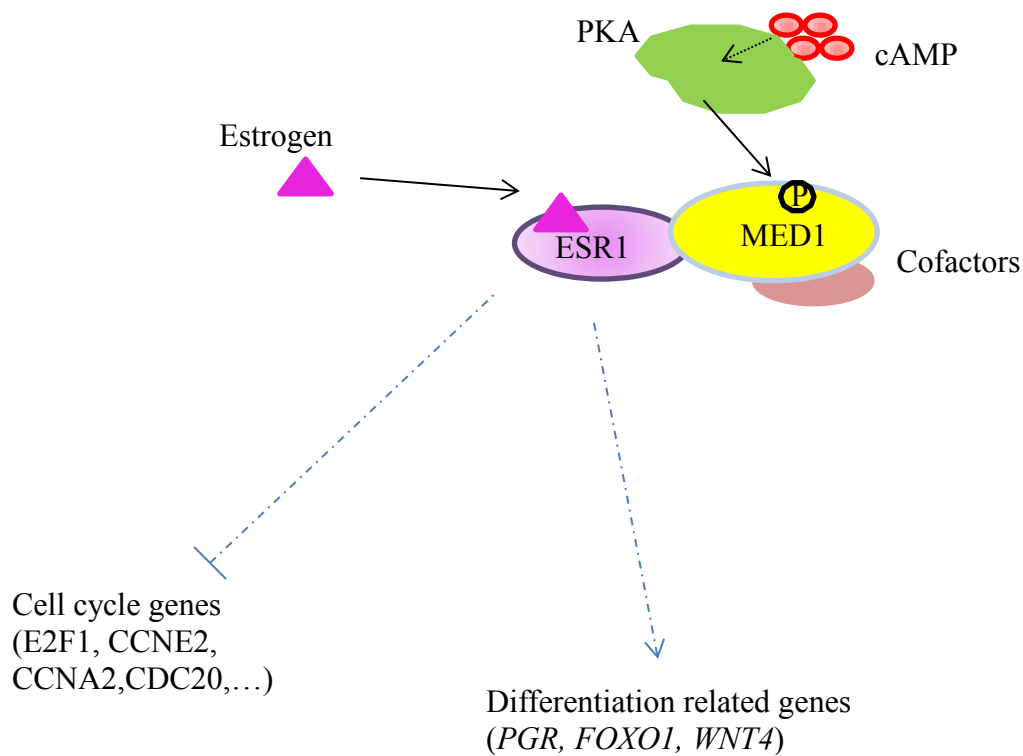


Figure 2.8: Proposed model for role of ESR1 and cAMP during HESC differentiation

ESR1 has two main roles in HESC differentiation: (i) it suppresses proliferation of HESC by suppressing the expression of cell cycle regulators; (ii) ESR1 induces the differentiation of HESC by inducing the expression of important regulators of HESC differentiation, namely *FOXO1*, *WNT4* and *PGR*. PKA signaling is the principal signaling pathway downstream of cAMP. MED1 is phosphorylated by PKA and interacts with ESR1 and is critical for HESC differentiation and expression of ESR1 target genes.

CHAPTER III

ROLE OF PROGESTERONE RECEPTOR ISOFORMS DURING HUMAN ENDOMETRIAL STROMAL CELL DIFFERENTIATION

ABSTRACT

Progesterone, acting through progesterone receptors (PRs), is known to be one of the most critical regulators of endometrial differentiation. Yet, a long-standing unresolved issue in uterine biology is the precise roles of the progesterone receptor isoforms, PRA and PRB, during endometrial differentiation in the human. Our approach, expressing PRA and PRB individually after silencing endogenous PRs in human endometrial stromal cells (HESC), enabled the analysis of the roles of the isoforms separately as well as jointly. We found that PRB has a larger cistrome, which includes the majority of PRA binding sites. *De novo* motif analysis showed that, while both isoforms bind to the same DNA sequence motif, they had both common and distinct nearby motifs where other transcription factors bind. Our comprehensive gene expression analysis suggested that PRA and PRB regulated overlapping and distinct sets of genes, many of which are critical for decidualization and establishment of pregnancy. When PRA and PRB were co-expressed, PRB was the prevalent isoform, although both isoforms influenced each other's transcriptional activity. Our ChIP-seq and genome-wide gene expression analyses suggested that PRB is a more effective transcription factor than PRA during HESC differentiation. With this study, we confirmed some of the known roles of progesterone, such as regulation of cell cycle, angiogenesis, apoptosis and expression of some essential signaling molecules, for stromal differentiation. We were also able to distinguish signaling networks downstream of each isoform and find novel molecules, such as IRS2, as potential direct targets of progesterone signaling to support a successful pregnancy.

INTRODUCTION

Decidualization, the differentiation of endometrium into a unique tissue, which supports the implanted embryo, is one of the most critical processes in the establishment of a successful

pregnancy. Decidualization is a complex event, every step of which is regulated by many transcription factors and signaling molecules in a timely manner. Progesterone (P), secreted from ovaries following ovulation, is one of the earliest and most important regulators of endometrial differentiation. It has been well established that P, acting through its nuclear receptors, PRs, is required for the precise and timely regulation of this process [1-6]. Several *in vitro* and *in vivo* studies, including characterization of the PR knockout mouse, have established a key role of progesterone receptor during decidualization in humans and mice [5] [7, 8].

Progesterone receptor has two isoforms, PRA and PRB, which are transcribed from the same gene [9]. Despite having the same DNA binding and ligand binding domains, PRA and PRB can have very different activities in the cell. The dissimilarities in their transcriptional activities originate from the differences in their N terminal regions, as PRB has an additional transactivation domain that interacts with cofactors [10]. A number of cell-based reporter assays suggested that PRA could act as a repressor of PRB transcriptional activity at some promoters [11, 12]. Studies in prostate stroma and breast cancer cell lines showed that PRA and PRB regulated different gene networks and their relative expression levels, which change in some breast cancer types, might affect downstream gene expression [13-15].

Both isoforms are expressed in the endometrial stroma and their relative levels change throughout the menstrual cycle [16]. In the early proliferative stage, PRB level is very low, while PRA levels are high. During the peri-ovulatory period, expression of both isoforms increase. The induction of PRB is more robust, its level becoming comparable to that of PRA. By late secretory phase, both PRA and PRB expression start to decline and their levels return to those seen in early proliferative phase [16, 17].

Some endometrial disorders show aberrant expression and relative ratios of PRA and PRB, underlining the importance of distinguishing between the roles of these isoforms so that we can have a better understanding of their biology, their role in gynecological pathologies, and to develop treatment strategies for diseases. Although, the molecular pathways via which the progesterone receptors exert their effects during decidualization have been addressed in some studies [6, 7, 18], we are still far away from understanding the downstream signaling mechanisms of each isoform during decidualization in the human.

Our approach, expressing PRA and PRB individually after silencing endogenous isoforms, allowed the identification of their genome-wide binding sites and downstream gene networks separately. Using this approach, we showed that they both bound to the same DNA sequence motif, but PRB had a larger number of binding sites. We also found that they had both common and unique neighboring motifs nearby where other transcription factors bind, suggesting the presence of different interaction partners. PRA and PRB showed common and distinct downstream gene networks when expressed individually. Signaling pathways identified downstream of the isoforms, including regulation of angiogenesis, apoptosis and cell cycle, are known to be critical components of the endometrial differentiation process. We also observed that PRA and PRB modulate each other's activity when they are co-expressed. Therefore we believe that our study, which aims to explore the roles of both isoforms during endometrial differentiation, is an important step for furthering our understanding of the roles of progesterone receptor isoforms in human uterine biology.

MATERIALS AND METHODS

Primary HESC culture

The primary human endometrial stromal cells (HESC) were provided by Dr. Robert Taylor of Emory University Medical School and Wake Forest School of Medicine. HESC were isolated from biopsies collected from proliferative stage endometrium of normal cycling women with no sign of uterine abnormality and with the volunteer's informed consent. These cells were cultured in DMEM/F-12 medium (Invitrogen) supplemented with 5% (v/v) fetal bovine serum (Hyclone), 50µg/ml penicillin and 50µg/ml streptomycin (Invitrogen). HESC of passage number nine or earlier were used in this study. For *in vitro* differentiation, the cells were treated with differentiation cocktail composed of 0.5mM 8-bromo-adenosine-3',5'-cyclic monophosphate (8-Br-cAMP) (Sigma), 1µM progesterone (Sigma) and 10nM 17-β-estradiol (Sigma) in DMEM/F-12 medium (Invitrogen) supplemented with 2% (v/v) charcoal dextran stripped fetal bovine serum. The medium/ differentiation cocktail was refreshed every 48h.

siRNA transfection and adenovirus transduction

HESC were transfected with siRNA targeting the 3'utr of progesterone receptor mRNA (Dharmacon) or non-targeting control siRNA (Dharmacon) following manufacturer's protocol (SilentFect, Bio-Rad Laboratories). Briefly, a final concentration of 50nM siRNA was mixed together with 5µl of SilentFect to transfect the cells. At 24h following siRNA treatment, cells were transduced with the adenovirus expressing flag tagged PRA or PRB for 24h. The adenovirus is kindly provided by laboratory of Prof. Dean Edwards of Baylor College of Medicine. At 48h of siRNA transfection and 24h of concurrent adenovirus transduction, medium was removed; cells were washed with PBS and treated with differentiation cocktail for time periods as indicated in the figure legends.

Western Blot Analysis

Primary HESCs were subjected to different treatments as indicated in the figure legends for indicated periods of time. Whole cell extracts were prepared and equal amounts of proteins were analyzed by SDS-PAGE, transferred to PVDF membrane and the specific proteins were detected by Western blotting using the antibody against Calnexin (Santa Cruz), and PR. PR antibody was kindly provided by Prof. Dean Edwards of Baylor College of Medicine.

Chromatin immunoprecipitation

ChIP assays were performed using the EZ-ChIP kit (Millipore) according to the manufacturer's instructions with minor modifications. HESC were seeded on 150mm dishes. After cells were attached, medium was switched to 2% (v/v) charcoal dextran stripped fetal bovine serum containing medium. When reached 70-80% confluency, cells were transfected with siRNA 24h and then transduced with adenovirus carrying flag-tagged PRA or PRB or empty vector (negative control) for 24h. Next day, cells were treated with E+P+8-Br-cAMP for 2h. Cells were fixed with 1% formaldehyde for 10 min and excess formaldehyde was quenched with 0.25 M glycine for 5 min. Cells were then washed twice with cold PBS, scraped and collected in PBS containing protease inhibitors in a conical tube, and centrifuged. Cell pellets were resuspended in lysis buffer containing protease inhibitors for 15 min to lyse the cells. Next, chromatin was sonicated in four 15-s pulses on power 4; with cooling between pulses (Fisher Scientific model

100 Sonic Dismembrator). One percent of the cell lysate was used for input control. The remainder of the lysate was incubated with anti-flag M2 affinity gel (Sigma, A2220) overnight at 4°C to isolate the immune complexes (flag-PRA and flag-PRB). For CEBPB ChIP, an antibody against CEBPB (Santa Cruz; sc-150) and for negative control mock IgG (Santa Cruz sc-2027) were used. Complexes were washed with low salt, high salt, LiCl buffers and twice with TE buffers, eluted from the beads and heated at 65°C for 6 hours to reverse the cross-linking. Following digestion with RNAase A and proteinase K, DNA fragments were purified using QIAquick PCR purification kit (Qiagen). For ChIP real-time quantitative PCR, primers were designed to amplify potential binding sites. The resulting signals were normalized to input DNA and relative fold enrichment was calculated as the enrichment in experimental samples over that of negative control ChIP.

Microarray sample preparation and data analysis

HESC were transfected with siRNA targeting *PGR* mRNA (Dharmacon) or non-targeting control siRNA (Dharmacon) as explained above. At 24h following siRNA treatment, cells were transduced with the adenovirus expressing flag tagged PRA, PRB or PRA-PRB both for 24h. Next day, cells were washed with PBS and treated with differentiation cocktail for 6h. We performed two independent microarray experiment sets using two different clinical samples. Total RNA was isolated by Trizol reagent (Invitrogen, CA) and further purified using RNeasy columns (Qiagen) following manufacturer's instructions. RNA samples were processed at the Biotechnology Center of the University of Illinois at Urbana-Champaign. RNA integrity was verified using Agilent 2100 Bioanalyzer. Each RNA sample was processed to generate labeled cRNA following established protocols for hybridization to the GeneChip Human Genome U133A 2 arrays. The resulting data files were first normalized and analyzed by Affymetrix GeneChip Expression Console software using RMA algorithm. Genes with a minimum of 1.2-fold-change in the same direction in both microarrays were considered as differentially regulated genes.

RNA Isolation and Real-time PCR Analysis

Total RNA was extracted from cultured HESC using Trizol (Invitrogen) according to manufacturer's instructions. Total RNA was subjected to cDNA conversion using Stratagene

cDNA conversion kit, following manufacturer's instructions. cDNA were subjected to quantitative PCR analysis using gene specific primers. Sequences of real time PCR primers are available upon request. 36B4 was used as the internal control. For a given sample, threshold cycle (Ct) and (standard deviation) SD was calculated from individual Ct values from three replicates of a sample. Normalized mean Ct (Δ Ct) was calculated by subtracting mean Ct of 36B4 from mean Ct of a target gene for control sample. $\Delta\Delta$ Ct was then calculated for each gene as a difference in Δ Ct values between control and sample. Fold change in gene expression was then computed as $2^{-\Delta\Delta\text{Ct}}$. Results are expressed as mean $\pm$ SD of three different measurements. Statistical significance between the control and experimental sample was determined using student t-test. A p-value of ≤ 0.05 was considered to be significant. Statistical significance within a group of experimental samples was determined using one-way ANOVA followed by Tukey mean comparison test. A p-value of < 0.05 was considered significant.

RESULTS

Roles of PRA and PRB can be analyzed individually during HESC differentiation

Undifferentiated HESCs express both isoforms of progesterone receptor at low levels. Upon addition of differentiation cocktail composed of estrogen (E), progesterone (P) and cAMP analog (cAMP), levels of both isoforms increase significantly, though more pronounced in PRB levels (Fig. 3.1A). After 24 h of hormone cocktail treatment, both isoforms are expressed at comparable levels in differentiating HESC. To analyze the roles of each isoform separately, we used a strategy to generate HESC expressing only one of the isoforms.. In our approach we transiently silenced the expression of endogenous receptor and then reconstituted the expression of each isoform individually by transient transduction with an adenovirus harboring the cDNA of the isoform. Endogenous PR was depleted in HESC by transfecting the cells with siRNA targeted to the 3'-untranslated region (UTR) of the PR mRNA. Next, cells were transduced with adenovirus harboring flag-tagged PRA or PRB cDNA lacking the 3' UTR or a control virus carrying the empty vector. This strategy allowed the reconstitution of PRA or PRB expression in the HESC and created conditions under which the biological activity of either PRA or PRB can be tested independently (Fig. 3.1B). To ascertain that the protein levels of exogenously expressed isoforms

are comparable to their endogenous levels, we employed western blot analysis using an antibody that recognizes both PRA and PRB (Fig. 3.1C). We next tested the biological activity of the flag-tagged PRA or PRB by monitoring their ability to rescue differentiation following the knock-down of endogenous PR. For this purpose, we monitored the expression of transcripts of PRL and IGFBP1, two well-known markers of stromal differentiation. In response to siRNA-mediated knockdown of endogenous PRA and PRB, the expressions of PRL and IGFBP1 were strongly down regulated (Fig. 3.1D). Reconstitution of PRB expression fully restored the IGFBP1 and PRL mRNA expression, while the rescue of the expression of these mRNAs by PRA was slightly less effective. These results support our view that adenovirus-induced flag-PRA and flag-PRB are biologically active and able to restore stromal differentiation, although they may differ in their inherent capacities to control this process.

Genome-wide PRA and PRB binding locations in – HESC during decidualization

In order to map the genomic binding sites of the P-R isoforms in differentiating HESC, we employed chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq). HESC depleted of endogenous P-R isoforms and expressing adenovirus-induced flag-PRA or flag-PRB were treated with differentiation cocktail for 2 h. ChIP assays were performed using an antibody specific for the flag epitope. Purified chromatin samples were sequenced using the Illumina platform. For mapping the Illumina sequencing reads to the human genome, we used the Bowtie software package [19]. Mapped reads were then analyzed with the MACs program [20] to identify peaks enriched in PRA ChIP or PRB ChIP samples compared to both genomic input and mock-ChIP controls. Mock-ChIP control was used as an internal control for validation of the specificity of the antibodies used for pulling down flag-tagged proteins. To identify genomic binding sites for each isoform, we used genomic input DNA comparison and for further analysis we considered sequences with a false discovery rate (FDR) $\leq 5\%$ and fold enrichment (FE) ≥ 5 . We identified 8248 genome-wide binding sites for PRA and 15109 for PRB. The majority of PRA binding sites were common to PRB, while PRB also occupied many unique binding sites (Fig. 3.2A). A subset of target sequences common to both PRA and PRB ChIP-seq data were selected for further validation by ChIP-RT-qPCR using different clinical samples (Fig. 3.2B). To further characterize the binding sites, we mapped the enriched regions identified by PRA and PRB ChIP-seq with custom defined genomic regions using CEAS (Enrichment on

chromosome and annotation) tool of Cistrome. We defined regions at 2500bp, 5000bp and 10000bp upstream of transcriptional start sites (TSS) as promoter – lower, -middle and –upper intervals. The majority of both PRA and PRB enriched regions were either distal intergenic regions or introns (Fig. 3.2C).

PRB regulates PRA

We identified two PR binding sites in the P-R gene itself. Interestingly, these sites were bound by PRB but not PRA. The binding site in the first exon of the P-R gene mostly overlapped with the sequence previously reported to be a PRA promoter (Fig. 3.2D) [9], suggesting that PRB might be binding to the PRA promoter to regulate its expression. We validated PRB binding to this region by ChIP, while PRA showed no enrichment (Fig. 3.2E). Since PRA shares a common coding sequence with PRB, it was not possible to monitor PRA mRNA levels independent of PRB mRNA to assess the possible transcriptional regulation of PRA by PRB. Therefore, we analyzed how the expression of exogenous PRA affects the PRB protein levels and vice versa. With increasing levels of exogenously expressed PRA, no noticeable increase in PRB protein levels was detected. In contrast, we observed a considerable increase in PRA protein levels in response to increasing levels of exogenously expressed PRB (Fig. 3.2F); consistent with the concept that PRB regulates PRA expression by directly binding to its promoter.

Analysis of transcription factor binding motifs associated with PRA and PRB binding sites

In order to identify enriched transcription factor binding motifs in the PR binding sites identified by ChIP-seq, we applied the *de novo* motif detection tools MEME [21] and SeqPos [22] to analyze sequences within ± 100 bp of the summits of PRA and PRB binding sites. Motif analysis of both PRA and PRB binding sites identified G.ACA...TGT.C as the major motif, which did not differ between the two isoforms (Fig. 3.3A). This motif, which is identical to the progesterone response element described previously [18] was located centrally within the 200bp sequence spanning the summit, (Fig. 3.3B). Figure 3.3C shows the number and overlap of additional motifs observed in each isoform's target sequences. A description of the motifs observed in the 200nt sequence around the summits of PRA and PRB binding sites are listed in Tables 3.1 and 3.2. We found several motifs common for both isoforms. One of these is an AR

binding motif, which is essentially the same as the PRE. It is known that PR and AR recognize the same binding motif. The other motifs represented the binding sites of various transcription factors, such as JUN, SOX10, CEBPA, CEBPB, FOXP1, HIF1A, REST, PAX1 and MTF1. The JUN motif was one of the most abundant motifs in both PRA and PRB target sequences. It was also identified as a prominent motif in the mouse PR cistrome [18]. Interestingly, JUN and FOS motifs were found more frequently in PRA bound sites than that of PRB. Motifs unique to PRA included HOXA2. Motifs unique to PRB included BCL6, STATs, TRP63 and RARA. In order to assess whether these nearby motifs were actual binding sites for corresponding transcription factors, we selected PRA and PRB binding sites that contained the CEBPB motif and analyzed the chromatin enrichment of this factor by ChIP. As shown in Fig. 3.3D, sequences which had CEBPB motif were significantly enriched, while there was no enrichment in a control sequence lacking CEBPB motif.

Genes regulated by PR isoforms in differentiating human endometrial stromal cells

The first step in understanding the molecular signaling downstream of each isoforms is to identify potential direct targets of PRA and PRB. We performed mRNA profiling to correlate genome binding to gene expression regulation. For the microarray analysis, HESC were transfected with control siRNA or with siRNA targeting 3'UTR of PR mRNA. SiRNA mediated knockdown was followed by transduction with adenovirus harboring flag-tagged PRA or PRB cDNA or a control virus carrying the empty vector. Importantly, we also co-expressed PRA and PRB to see whether the presence of one isoform influences the transcriptional activity of the other. Our analysis yielded 451, 1724, and 1636 differentially regulated genes in HESC expressing PRA alone, PRB alone and PRA+PRB, respectively, compared with HESC transduced with control virus. We next assessed the percentage of PRA or PRB upregulated and downregulated genes that had at least one PRA or PRB-binding site within ± 100 kb of their TSSs. About 40% of genes regulated by PRA had a PRA binding site while 60% of genes regulated by PRB contained a PRB binding site (Fig. 3.4D and Fig. 3.4E). We verified that several genes previously reported to be progesterone-regulated indeed possess PRA or PRB binding sites and their expression was regulated by progesterone. , For example, the BMP2 gene [23], displayed multiple binding sites for both PRA and PRB and its expression was increased by PRB at 6 h. We also examined whether the neighboring motifs determine the direction of

regulation of target genes. We noted that, in PRB binding sites of down regulated genes, STAT and REST motifs were enriched, while CEBP, HIF1A and ELK motifs were enriched in the PRB binding sites of up regulated genes. To have a better understanding of the key biological functions associated with the gene pathways downstream of PRA and PRB, we classified them into various biological categories, using DAVID [24, 25] and PANTHER.

We found that about 25% of genes regulated by PRA overlapped with PRB-regulated genes (Fig. 3.4A). These common pathways included genes that regulate response to external stimulus, blood vessel development, regulation of blood coagulation, and response to hypoxia.

PRA-regulated genes encoded several protein kinases, and transcription factors involved in the regulation of apoptosis (including *TNFSF12*, *BCL6*, *CFLAR*, *MCL1* and *BIRC5*), and factors involved in blood vessel development (including *CYR61* and *VEGFA*) (Table 3.3).

Our analysis highlighted the predominant roles of PRB in the regulation of the cell cycle, angiogenesis, lysosomal activity and secretion of chemokines and cytokines (Table 3.4). Genes upregulated by PRB included several signaling molecules involved in angiogenesis, apoptosis and cell adhesion, and numerous solute carrier family members. Interestingly, many genes regulating the mitotic phase of the cell cycle, including *CCNB1*, *CCNB2*, *CDC20*, *CDC25A*, *CDK1* and *BUB1*, were suppressed by PRB (Fig 3.5A), suggesting that the PRB isoform may be playing a critical role in suppression of cell proliferation during the differentiation process of HESC. It is generally observed that the cell cycle exit is a pre-requisite for differentiation. Our laboratory and others have observed that proliferation of HESC ceased at the initiation of the differentiation program [26]. The exit from the cell cycle is a key step in HESC differentiation and it is potentially regulated by PRB.

Angiogenesis appears to be another biological process regulated by PRB. We found several genes, including *VEGFA*, *ANGPT2*, *ANGPTL4*, *EPAS1*, *EREG*, *TGFB2*, *EGFR*, *FGF2* and *TNFAIP2* that are up-regulated by PRB. Formation of new maternal blood vessels is critical for implantation and survival of the embryo, and progesterone is known to stimulate angiogenesis during decidualization. Many of these genes, such as *VEGFA*, *TGFB2*, *ANGPT2*, *ANGPTL4* and *EGFR*, harbor PRB binding sites, indicating that they are direct targets of PRB. We validated the induction of several of these genes by PRB using real-time PCR (Fig. 3.5B).

When both isoforms were co-expressed, differentially regulated genes were more commonly associated with PRB (Table 3.5, Fig. 3.4B, 3.4C). However, PRA clearly modulated PRB activity as the magnitudes of expression of several genes were altered when both isoforms were co-expressed. These genes included numerous glycoproteins involved in cell signaling and immunoregulation, ion channels, peroxisome-related genes, and certain peptidases involved in lysosomal processes.

IRS2, a PR-regulated metabolic molecule, controls decidualization

We identified several components of the insulin signaling pathway, including insulin receptor substrate 2 (*IRS2*), SHC-transforming proteins 1 and 3 (*SHC1* and *SHC3*), *GRB10* and *SOS1* downstream of PRB. Among them, *IRS2* contained binding sites for both PRA and PRB at 88kb upstream of its TSS, in addition to several other binding sites farther upstream or downstream of its open reading frame. We monitored *IRS2* expression in primary cultures of differentiating HESC. Addition of differentiation cocktail led to an increase in *IRS2* mRNA levels within 24 h (data not shown). To establish *IRS2* as a direct target of PR, we validated the binding of PRA and PRB to the -88kb upstream region and an additional region further upstream. Both PRA and PRB were recruited to these regions (Fig 3.6A). Next, we confirmed transcriptional regulation of *IRS2* by PRA and PRB at 24 h of HESC differentiation. When endogenous PR was silenced by siRNA targeting 3'utr region of its mRNA, *IRS2* expression was also downregulated. Reconstitution of PRA and PRB expression restored the *IRS2* mRNA expression (Fig. 3.6B). To test the possibility of a potential role of *IRS2* in HESC differentiation, we examined the effect of this down regulation on the differentiation process. Analysis of mRNAs corresponding to *PRL*, *IGFBP1* and *WNT4*, well-known biochemical markers of decidualization, by real-time RT-PCR, revealed a significant decrease in the expression of *IGFBP1* and *WNT4* (Fig. 3.6C). Similar to the gene expression changes, indicating a lack of stromal differentiation in the absence of *IRS2*, there was impairment in the morphological changes associated with the decidualization process (data not shown). Therefore, we concluded that *IRS2* is a potential direct target of progesterone receptor and is critical for the differentiation of HESC.

DISCUSSION

In the present study, we aimed to identify the downstream gene networks and potential direct targets of progesterone receptor isoforms, PRA and PRB, during human stromal differentiation. Both isoforms were expressed at comparable levels in differentiating HESC (Fig. 3.1A) and capable of inducing stromal decidualization as measured by the expression of classical decidualization biomarkers, IGFBP1 and PRL (Fig. 3.1D), suggesting that both isoforms played critical regulatory roles during stromal differentiation.

Since they are expressed from the same gene, PRA and PRB have the same DNA binding and ligand binding domains, but they have very different biological activities due to the differences in their N terminal regions. PRB has a B-upstream segment (BUS) which can interact with adjacent PRB dimers, different transcription factors and co-activators [10, 11, 27]. By our unbiased genome-wide approach, we identified cistromes of PRA and PRB at an early time point of *in vitro* differentiation. We found that PRB had a greater number of binding regions in the genome, including the majority of the PRA binding sites (Fig. 3.2A). This suggested that PRB might have a larger transcriptome compared to PRA. There are several possible mechanisms by which this may happen, including cooperative interactions between PRB homodimers occupying multiple adjacent PR binding sites and interactions with other factors that bind to nearby motifs. When we analyzed the motifs within a ± 100 bp window from the summits of target sequences of PRA and PRB, we identified motifs that were unique to PRA (BACHs and HOXA2) and PRB (BCL6, STATs, TRP63, and RARA). In addition, PRA and PRB had common nearby including JUN, CEBPs, SOXs, REST and ESR1, some of which had been observed previously in the mouse PR cistrome [18].

These motifs, which bind to other transcription factors, may serve as indirect binding sites for PRA or PRB through tethering mechanisms. Consistent with this view, it has been proposed that certain pioneering transcription factors modulate chromatin availability for PRs [28]. Alternatively, PRA or PRB, bound directly to target DNA, may interact with other transcription factors, such as CEBPB, STAT3, and HIF1A, and these interactions might be critical for a cooperative regulation of target gene expression. This adds another level of complexity to the pattern of temporal regulation of PR target genes, since activation and nuclear localization of

some of these factors occur hours to days after differentiation stimulus. For example, we observed nuclear localization of STAT3 in HESC 24 hours after stimulation with the differentiation cocktail [26]. Notably, these findings suggest that PR can potentially undergo direct protein-protein interactions with previously identified critical regulators of stromal differentiation, including CEBPB, HOXA10, STATs, HIF1A and FOXO [26, 29-31]. Previous studies speculated that PR undergoes functional interactions with FOXO1, STAT3 and CEBPB to regulate the expression of target genes in differentiating stromal cells [30-32]. In support of this theory, we showed a significant enrichment of CEBPB on several progesterone receptor binding sites identified in our study, suggesting that coordinate gene regulation involving PR and these transcription factors are critical for target gene expression and decidualization.

Similar to other steroid receptors [33, 34], binding sites for the PR isoforms were localized further away from the TSS of the closest genes, mostly in introns or intergenic regions (Fig. 3.2B). Further characterization revealed that about 80% of all binding regions overlapped with enhancer- promoter- associated histone marks (layered H3K4Me1 and hypersensitive sites) [35], resembling distant enhancers (data not shown). For example, we identified multiple PRB binding sites, scattered in the region where all 11 members of the family of pregnancy specific glycoproteins (PSGs) are clustered in chromosome 19 [36] (Fig. 3.7A). The majority of these binding sequences corresponded with layered H3K4Me1 available in UCSC Genome Browser [37, 38]. Our microarray results confirmed that the - expression- of several PSGs, including PSG1, PSG2, PSG4, PSG5, PSG7 and PSG9, is regulated by PRB. We selected PSG4 as a representative gene among this cluster and validated progesterone receptor enrichment by ChIP and its up-regulation by PRB, using real-time PCR (Fig. 3.7B and 3.7C). Since there were multiple PRB binding sites around PSG4, it gave us an opportunity to test the possible functional role of the distal binding sites. For this purpose, we employed chromosome conformation capture (3C) assay on two PRB binding sites; one between exon 3 and exon 4 of PSG4 and another located about 80kb upstream of PSG4 TSS. Our aim was to examine possible interaction between a distant binding site located in an enhancer-like region and proximal binding sites/promoter of a PR target gene-. Remarkably, our results were consistent with an interaction between these two binding sites in HESC expressing PRB and this interaction was seen only in the presence of the differentiation cocktail (Fig. 3.7D). This experiment- indicated that progesterone receptor bound to distal sites may facilitate transcription of its target gene by a

looping mechanism. It is conceivable that, in uterine tissue, both PRA and PRB regulate the expression of the majority of their target genes through long-range enhancer interactions rather than binding to promoter proximal regions of the target genes.

Our findings are consistent with the idea that other factors might be involved in regulation of gene expression by PR isoforms. Gene expression analysis during HESC differentiation revealed that only a small fraction of downstream genes of PRA and PRB was common. We believe that interaction of PRA and PRB with specific nearby motifs, such as STATs for PRB and HOXAs for PRA, might contribute to the differences we see in the downstream gene networks. STATs have been reported to be critical for HESC differentiation, presumably via direct interactions with PRB [39]. Interestingly, when we grouped genes downstream of PRB according to their direction of regulation, the REST (RE1-Silencing Transcription factor) motif was enriched more in repressed genes while CEBPB, HIF1, ELKx and ETVx motifs were found more in upregulated genes, suggesting that the presence of specific transcription factors near a PR binding site may dictate the direction of regulation of the target gene.

Our gene expression analysis, using HESC expressing PRA, PRB, or a combination of PRA and PRB, provided a comprehensive view of the pathways regulated by progesterone signaling during decidualization. These included (i) the distinct pathways regulated by each receptor isoform, (ii) common pathways regulated by both, and (iii) unique pathways that were regulated when both receptor isoforms were present. More than half of the genes found downstream of PRA and PRB at 6 h of differentiation contained at least one PRA or PRB binding site within 100kb from its TSS, suggesting that they may be direct targets of progesterone signaling. Several of these genes, such as BMP2, STAT3, WNT4, HOXA10, and HAND2 are critical for HESC differentiation [40]. Many of the potential direct targets of both PRA and PRB were found to be involved in regulation of angiogenesis, immune response, cell cycle, apoptosis and cell-matrix interactions. We believe that all of these functional categories are critical for decidualization and maintenance of pregnancy. We will briefly discuss some of these critical biological processes.

Cell cycle regulation: Our microarray analysis revealed that, in differentiating HESC, many genes involved in M phase of the cell cycle, including CCNB2, CDC25A, CDC25C, CDK1 and AURKA, were repressed in HESC expressing either PRB and PRA+PRB. It is generally

observed that the cell cycle exit is needed for differentiation [41]. We and others have observed that HESC, which proliferate well in the growth medium, ceased proliferation with the addition of differentiation cocktail [26]. We suggest that PRB may be contributing to the transition of HESC from a proliferating state to a differentiating state by suppressing G2M progression. , The biological relevance of this is found in the increase in PRB expression in human endometrial stromal cells at the periovulatory period as they begin to differentiate and transit from a proliferative to a secretory phase of the cycle.

Angiogenesis: Angiogenesis is essential for normal endometrial development and for placentation. New blood vessels must be formed in the decidua during pregnancy to support the developing embryo. Many diseases of pregnancy, such as premature termination and fetal growth restriction, have been associated with abnormal angiogenesis[42]. Our e gene expression profiling analysis showed that although both PRA and PRB regulate several genes involved in angiogenesis, PRB was the predominant regulator of this process. Angiogenesis related genes found downstream of PRB included VEGFA, ANGPT2, ANGPTL4 and FGF2. Among them, ANGPT2, ANGPTL4 and FGF2 contained PRB binding sites upstream of their TSS, suggesting that they are direct targets of PRB. The vascular endothelial growth factor (VEGFA), a key angiogenic factor in human endometrium, was previously shown to be regulated by progesterone in the uterus [43]. In our study, we found VEGFA transcripts to be induced by PRB at 6 h after initiation of differentiation. Although we did not find any PRB binding sites within a ± 100 kb window of the VEGF TSS, we identified several binding sites within a ± 200 kb window. We speculate that these regions might be bound by PRB, which then regulates VEGFA expression through a looping mechanism. Further research is necessary to understand regulation of VEGFA by PRB.

Lysosomal proteins: One of the most prominent functional categories of genes upregulated by PRB was lysosomal proteins, including acid phosphatases, cathepsins, arylsulfatase, lysosomal-associated membrane proteins, and solute carrier proteins. Changes in lysosomal enzyme activities, including increases in acid phosphatase and arylsulfatase B activity during initial stages of decidualization were reported previously [44]. These enzymes were maintained in lysosomes as progesterone stabilized the lysosomal membranes during decidualization., Progesterone withdrawal during menstruation resulted in the release of these enzyme [45]. We

believe that future studies will help us understand the roles of these lysosomal enzymes, structural lysosomal membrane proteins, and carrier proteins during decidualization and pregnancy.

Apoptosis: According to our gene expression analysis, apoptosis is one of the most prominent biological processes regulated by PRA. Apoptosis is normally observed during the menstrual cycle. During the early and late proliferative phase, there is little to no apoptosis; by the late secretory phase, marked apoptosis is seen and it progresses through most of the functional layer of endometrium [46]. Compared to undifferentiated HESCs, differentiating cells were reported to be resistant to oxidative stress-induced apoptosis [47], suggesting that resistance to oxidative stress might be an acquired feature of decidualized HESC and may be a part of the differentiation process itself. Interestingly, we found both negative (BIRC5 and CFLAR) and positive (SMAD3 and TGFB2) regulators of apoptosis downstream of PRA, suggesting that PRA might be involved in regulating the balance of these factors to control apoptosis appropriately in response to various internal and external signals.

Pregnancy specific glycoproteins (PSGs): As mentioned previously, several PSGs were upregulated by PRB in our microarray analysis. The PSG proteins are reported to be expressed by placental trophoblasts. However, low PSG11 expression in uterine endometrium during the peri-implantation period has been correlated with increased risk of pregnancy loss, suggesting that PSGs expressed by the endometrium may also have critical functions [48]. Although their biological functions are not fully understood, recent studies suggest that these glycoproteins may play important immunomodulatory roles to protect the embryo from maternal immune response [49]. Additionally, they may be involved in the formation of new vasculature needed for successful implantation [50]. We speculate that PSGs may be secreted by decidual cells downstream of PRB to regulate the action of cytokines, contribute to immunomodulation, and aid in blood vessel formation until trophoblasts take over the PSG production.

Solute carrier proteins: We found several solute carrier proteins, including monoamine transporters, glucose transporters, and ion exchangers downstream of PRB and PRA-PRB co-expressing cells. These molecules may be critical for monoamine metabolism, which is important for regulation of blood flow and capillary permeability [51] and nutrient/ion transport.

Further research is necessary to appreciate the biological roles of these transporter proteins in decidualization and reproductive physiology.

IRS2 signaling: IRS2 was shown previously to be regulated by progesterone [45]. Our study confirms these findings and further suggests that IRS2 is a direct target gene of both PRA and PRB. This molecule was of particular interest since IRS2, together with IRS1 and SHC1, is a key mediator for the downstream signaling pathways of both insulin and insulin like growth factor 1 (IGF-1). The expression and phosphorylation status of IGF system components were studied in human decidualization before; both expression and phosphorylation of IRS2 were reported to be increased in *in vitro* decidualized cells [52], suggesting that it might be a critical player of the differentiation process. In this study, we showed that both PRA and PRB were recruited to the enhancer like regions upstream of IRS2 and increased its expression. Importantly, we recognized that IRS2 expression was necessary for human decidualization since *in vitro* differentiation was impaired when IRS2 was silenced. This finding suggested that IRS2 might act as a link between insulin/IGF1 and progesterone signaling and potentially implicated aberrant IRS2 signaling in reproductive disorders associated with insulin metabolism [52, 53].

Our study is unique in terms of identifying cistromes and correlated gene expression profiles of PRA and PRB in differentiating human endometrial stromal cells. Our findings suggested that PRB was a more potent transcription factor than PRA in human stromal cell differentiation in terms of the extent to which it interacts with the genomic binding sites and regulates downstream genes. We also found that when PRA and PRB are co-expressed, PRB was the prevalent isoform, while PRA was able to influence PRB's transcriptional activity in some cases. This study provided a molecular understanding of some of the known roles of progesterone, such as regulation of cell cycle, angiogenesis, and apoptosis. We were also able to identify unique target pathways, such as IRS2, downstream of progesterone signaling that is likely to play a critical role in pregnancy.

REFERENCES

1. Lydon, J.P., et al., *Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities*. Genes Dev, 1995. 9(18): p. 2266-78.
2. Mulac-Jericevic, B. and O.M. Conneely, *Reproductive tissue-selective actions of progesterone receptors*. Ernst Schering Res Found Workshop, 2005(52): p. 19-37.
3. Franco, H.L., et al., *In vivo analysis of progesterone receptor action in the uterus during embryo implantation*. Semin Cell Dev Biol, 2008. 19(2): p. 178-86.
4. Brosens, J.J. and B. Gellersen, *Death or survival--progesterone-dependent cell fate decisions in the human endometrial stroma*. J Mol Endocrinol, 2006. 36(3): p. 389-98.
5. Conneely, O.M., et al., *Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice*. Mol Cell Endocrinol, 2001. 179(1-2): p. 97-103.
6. Popovici, R.M., L.C. Kao, and L.C. Giudice, *Discovery of new inducible genes in in vitro decidualized human endometrial stromal cells using microarray technology*. Endocrinology, 2000. 141(9): p. 3510-3.
7. Cloke, B., et al., *The androgen and progesterone receptors regulate distinct gene networks and cellular functions in decidualizing endometrium*. Endocrinology, 2008. 149(9): p. 4462-74.
8. Brosens, J.J., N. Hayashi, and J.O. White, *Progesterone receptor regulates decidual prolactin expression in differentiating human endometrial stromal cells*. Endocrinology, 1999. 140(10): p. 4809-20.
9. Kastner, P., et al., *Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B*. EMBO J, 1990. 9(5): p. 1603-14.
10. Conneely, O.M., B. Mulac-Jericevic, and J.P. Lydon, *Progesterone-dependent regulation of female reproductive activity by two distinct progesterone receptor isoforms*. Steroids, 2003. 68(10-13): p. 771-8.

11. Vegeto, E., et al., *Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function*. Mol Endocrinol, 1993. 7(10): p. 1244-55.
12. Wen, D.X., et al., *The A and B isoforms of the human progesterone receptor operate through distinct signaling pathways within target cells*. Mol Cell Biol, 1994. 14(12): p. 8356-64.
13. Khan, J.A., et al., *Differential regulation of breast cancer-associated genes by progesterone receptor isoforms PRA and PRB in a new bi-inducible breast cancer cell line*. PLoS One, 2012. 7(9): p. e45993.
14. Yu, Y., et al., *Expression and function of the progesterone receptor in human prostate stroma provide novel insights to cell proliferation control*. J Clin Endocrinol Metab, 2013. 98(7): p. 2887-96.
15. Richer, J.K., et al., *Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells*. J Biol Chem, 2002. 277(7): p. 5209-18.
16. Mote, P.A., et al., *Colocalization of progesterone receptors A and B by dual immunofluorescent histochemistry in human endometrium during the menstrual cycle*. J Clin Endocrinol Metab, 1999. 84(8): p. 2963-71.
17. Mangal, R.K., et al., *Differential expression of uterine progesterone receptor forms A and B during the menstrual cycle*. J Steroid Biochem Mol Biol, 1997. 63(4-6): p. 195-202.
18. Rubel, C.A., et al., *Research resource: Genome-wide profiling of progesterone receptor binding in the mouse uterus*. Mol Endocrinol, 2012. 26(8): p. 1428-42.
19. Langmead, B., et al., *Ultrafast and memory-efficient alignment of short DNA sequences to the human genome*. Genome Biol, 2009. 10(3): p. R25.
20. Zhang, Y., et al., *Model-based analysis of ChIP-Seq (MACS)*. Genome Biol, 2008. 9(9): p. R137.
21. Bailey, T.L., et al., *MEME SUITE: tools for motif discovery and searching*. Nucleic Acids Res, 2009. 37(Web Server issue): p. W202-8.

22. Liu, T., et al., *Cistrome: an integrative platform for transcriptional regulation studies*. Genome Biol, 2011. 12(8): p. R83.
23. Li, Q., et al., *Bone morphogenetic protein 2 functions via a conserved signaling pathway involving Wnt4 to regulate uterine decidualization in the mouse and the human*. J Biol Chem, 2007. 282(43): p. 31725-32.
24. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists*. Nucleic Acids Res, 2009. 37(1): p. 1-13.
25. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources*. Nat Protoc, 2009. 4(1): p. 44-57.
26. Wang, W., et al., *Regulation of human endometrial stromal proliferation and differentiation by C/EBPbeta involves cyclin E-cdk2 and STAT3*. Mol Endocrinol, 2012. 26(12): p. 2016-30.
27. Tung, L., et al., *Progesterone receptors (PR)-B and -A regulate transcription by different mechanisms: AF-3 exerts regulatory control over coactivator binding to PR-B*. Mol Endocrinol, 2006. 20(11): p. 2656-70.
28. Ballare, C., et al., *Nucleosome-driven transcription factor binding and gene regulation*. Mol Cell, 2013. 49(1): p. 67-79.
29. Wang, W., et al., *The CCAAT/enhancer binding protein beta is a critical regulator of steroid-induced mitotic expansion of uterine stromal cells during decidualization*. Endocrinology, 2010. 151(8): p. 3929-40.
30. Lee, J.H., et al., *Signal transducer and activator of transcription-3 (Stat3) plays a critical role in implantation via progesterone receptor in uterus*. FASEB J, 2013. 27(7): p. 2553-63.
31. Takano, M., et al., *Transcriptional cross talk between the forkhead transcription factor forkhead box O1A and the progesterone receptor coordinates cell cycle regulation and differentiation in human endometrial stromal cells*. Mol Endocrinol, 2007. 21(10): p. 2334-49.

32. Christian, M., et al., *Functional association of PR and CCAAT/enhancer-binding protein beta isoforms: promoter-dependent cooperation between PR-B and liver-enriched inhibitory protein, or liver-enriched activatory protein and PR-A in human endometrial stromal cells*. Mol Endocrinol, 2002. 16(1): p. 141-54.
33. Welboren, W.J., et al., *ChIP-Seq of ERalpha and RNA polymerase II defines genes differentially responding to ligands*. EMBO J, 2009. 28(10): p. 1418-28.
34. Carroll, J.S., et al., *Genome-wide analysis of estrogen receptor binding sites*. Nat Genet, 2006. 38(11): p. 1289-97.
35. Rosenbloom, K.R., et al., *ENCODE data in the UCSC Genome Browser: year 5 update*. Nucleic Acids Res, 2013. 41(Database issue): p. D56-63.
36. Teglund, S., et al., *The pregnancy-specific glycoprotein (PSG) gene cluster on human chromosome 19: fine structure of the 11 PSG genes and identification of 6 new genes forming a third subgroup within the carcinoembryonic antigen (CEA) family*. Genomics, 1994. 23(3): p. 669-84.
37. Kent, W.J., et al., *The human genome browser at UCSC*. Genome Res, 2002. 12(6): p. 996-1006.
38. Karolchik, D., et al., *The UCSC Table Browser data retrieval tool*. Nucleic Acids Res, 2004. 32(Database issue): p. D493-6.
39. Nagashima, T., et al., *Activation of SRC kinase and phosphorylation of signal transducer and activator of transcription-5 are required for decidual transformation of human endometrial stromal cells*. Endocrinology, 2008. 149(3): p. 1227-34.
40. Li, Q., et al., *WNT4 acts downstream of BMP2 and functions via beta-catenin signaling pathway to regulate human endometrial stromal cell differentiation*. Endocrinology, 2013. 154(1): p. 446-57.
41. Miller, J.P., et al., *Interweaving the cell cycle machinery with cell differentiation*. Cell Cycle, 2007. 6(23): p. 2932-8.
42. Reynolds, L.P., S.D. Killilea, and D.A. Redmer, *Angiogenesis in the female reproductive system*. FASEB J, 1992. 6(3): p. 886-92.

43. Ancelin, M., et al., *A dynamic shift of VEGF isoforms with a transient and selective progesterone-induced expression of VEGF189 regulates angiogenesis and vascular permeability in human uterus*. Proc Natl Acad Sci U S A, 2002. 99(9): p. 6023-8.
44. Moulton, B.C., B.B. Koenig, and S.C. Borkan, *Uterine lysosomal enzyme activity during ovum implantation and early decidualization*. Biol Reprod, 1978. 19(1): p. 167-70.
45. Fritz, M.A. and L. Speroff, *Clinical gynecologic endocrinology and infertility*. 8th ed. 2011, Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins. x, 1439 p.
46. Shikone, T., et al., *Apoptosis of human ovary and uterine endometrium during the menstrual cycle*. Horm Res, 1997. 48 Suppl 3: p. 27-34.
47. Kajihara, T., et al., *Human chorionic gonadotropin confers resistance to oxidative stress-induced apoptosis in decidualizing human endometrial stromal cells*. Fertil Steril, 2011. 95(4): p. 1302-7.
48. Arnold, L.L., et al., *Pregnancy-specific glycoprotein gene expression in recurrent aborters: a potential correlation to interleukin-10 expression*. Am J Reprod Immunol, 1999. 41(3): p. 174-82.
49. Snyder, S.K., et al., *Pregnancy-specific glycoproteins function as immunomodulators by inducing secretion of IL-10, IL-6 and TGF-beta1 by human monocytes*. Am J Reprod Immunol, 2001. 45(4): p. 205-16.
50. Blois, S.M., et al., *Early expression of pregnancy-specific glycoprotein 22 (PSG22) by trophoblast cells modulates angiogenesis in mice*. Biol Reprod, 2012. 86(6): p. 191.
51. Hansson, S.R., et al., *Monoamine transporters in human endometrium and decidua*. Hum Reprod Update, 2009. 15(2): p. 249-60.
52. Ganef, C., et al., *The IGF system in in-vitro human decidualization*. Mol Hum Reprod, 2009. 15(1): p. 27-38.
53. Burks, D.J., et al., *IRS-2 pathways integrate female reproduction and energy homeostasis*. Nature, 2000. 407(6802): p. 377-82.

54. Miele, A., Gheldof, N., Tabuchi, T.M., Dostie, J., and Dekker, J. (2006). Mapping chromatin interactions by chromosome conformation capture. *Curr. Protoc. Mol. Biol.* 74, 21.11.1–21.11.20

FIGURES AND TABLES

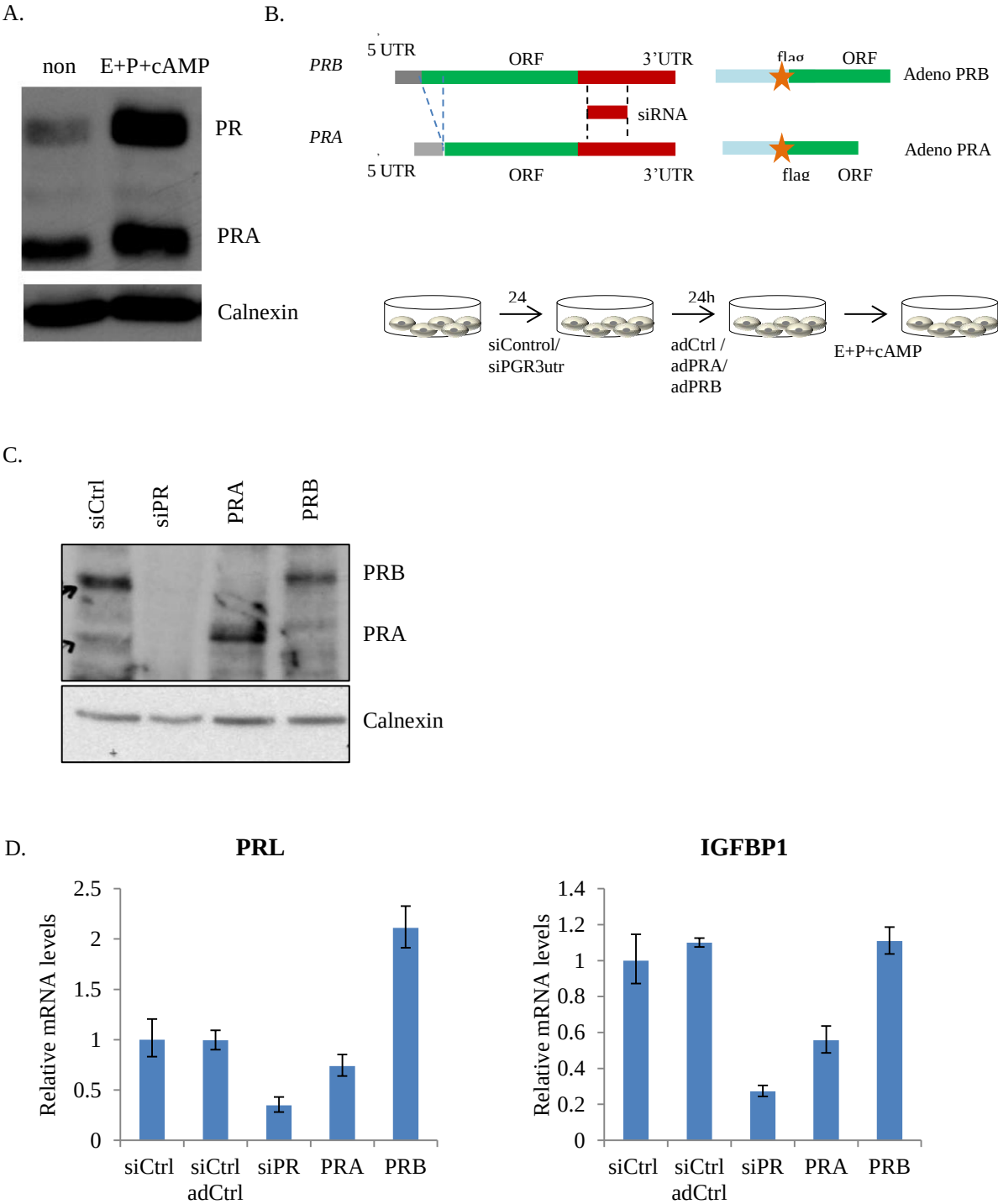


Figure 3.1: Analysis of individual roles of PRA and PRB in differentiating HESC

Figure 3.1 cont.:

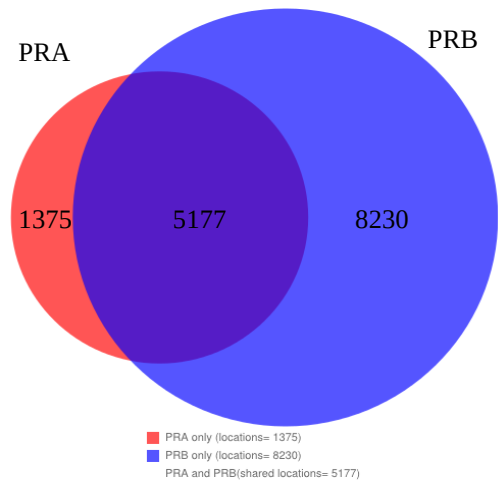
A. HESC were either untreated or treated with differentiation cocktail (E+P+cAMP) as indicated. Cells were lysed at 24h following treatment, analyzed by SDS-PAGE and subjected to Western Blotting using a monoclonal antibody that recognizes both PRA and PRB (kindly provided by Dr. Dean Edwards of Baylor College of Medicine) and Calnexin antibody. Calnexin immunostaining served as a loading control. Data is representative of five independent experiments.

B. Endogenous PR was depleted in HESC by transfecting the cells with siRNA targeted to the 3'-untranslated region (UTR) of the PGR mRNA for 24h. Next, cells were transduced with adenovirus harboring flag-tagged PRA or PRB cDNA without the 3' UTR or a control virus carrying the empty vector for 24h. This strategy allowed the reconstitution of PRA or PRB expression in the HESC and created conditions under which the biological activity of either PRA or PRB can be tested independently.

C. HESC were treated with 50nM control siRNA or siRNA targeting 3'UTR of PR mRNA. Next, cells were transduced with adenovirus harboring flag-tagged PRA or PRB or control virus for 24h. Whole cell lysates were prepared from these cells. For each sample, 30µg of whole cell lysate was analyzed by western blotting using a monoclonal antibody that recognizes both PRA and PRB. Immunostaining of calnexin was used as a loading control.

D. HESC were treated with 50nM control siRNA or siRNA targeting 3'UTR of PR mRNA. Next, cells were transduced with adenovirus harboring flag-tagged PRA or PRB or control virus for 24h. Then, siRNA and adenovirus were removed and cells were treated with differentiation cocktail. Total RNA was isolated at 60 h after differentiation cocktail addition and cDNAs were prepared. cDNAs were subjected to real-time PCR using gene specific primers for PR, PRL, and IGFBP1. PCR data are normalized to 36B4 mRNA levels. Relative fold changes were normalized with respect to control siRNA treatment.

A.



B.

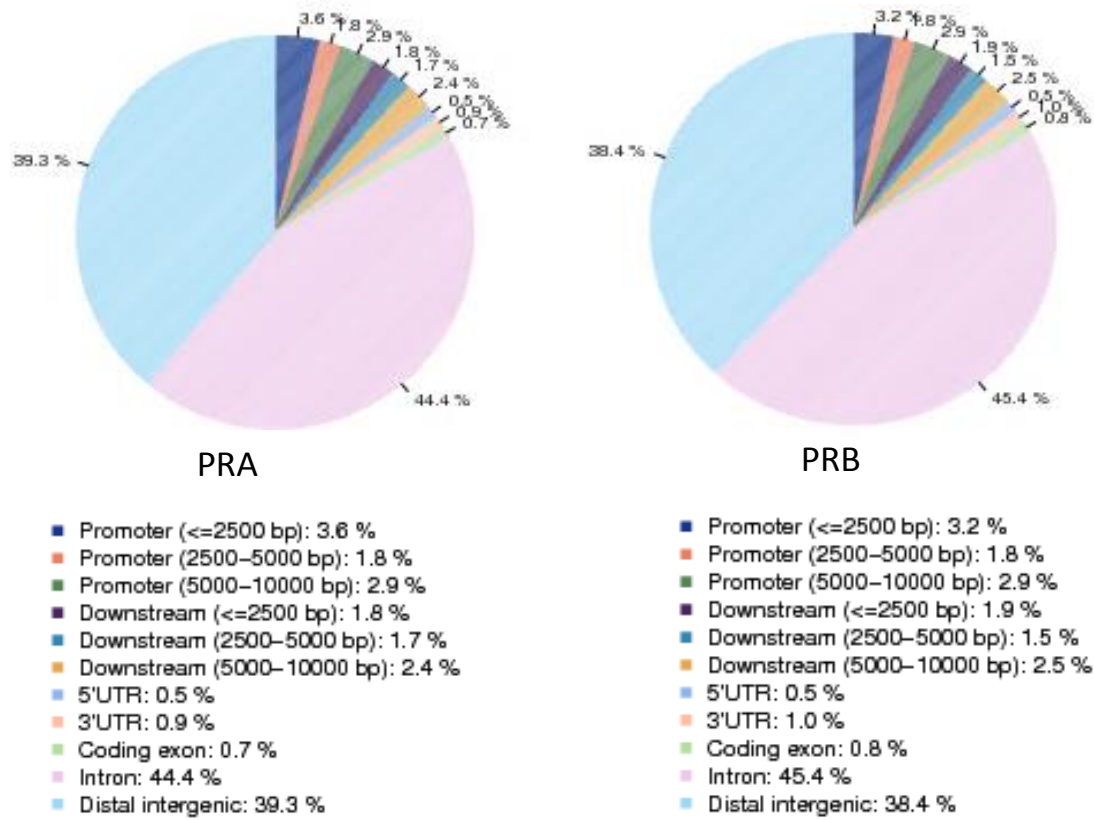


Figure 3.2: Genome-wide PRA and PRB binding locations in differentiating HESC

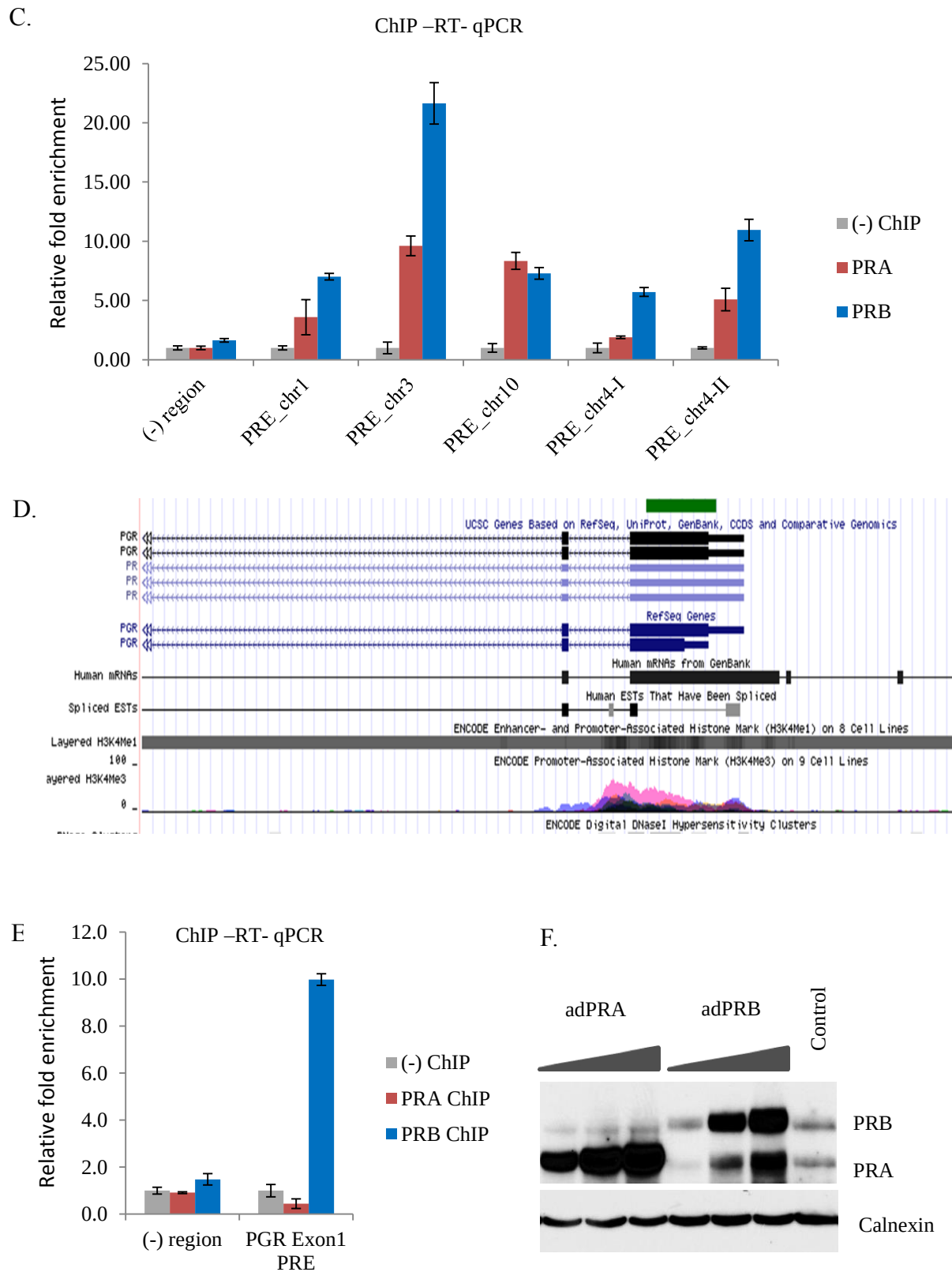


Fig. 3.2 cont.

Fig. 3.2 cont.:

- A. ChIP-seq PRA and PRB binding site counts. Venn diagrams of the intersection of multiple intervals files between PRA vs. PRB binding sites with $\text{fdr} < 5\%$ cut off.
- B. A subset of progesterone receptor binding sites identified in the ChIP-seq experiment was selected for validation. ChIP was performed as described in materials and methods section. Chromatin enrichment was quantified by real-time PCR using primers flanking these potential binding sites on different chromosomes as indicated in the figure. Negative region was a PRE deficient region. To calculate the enrichment of chromatin, the resulting signals were normalized to 1% input DNA. Data are represented as fold enrichment over that of the negative control ChIP. Data is representative of three independent experiments.
- C. Binding site- genomic region association of genome-wide PRA and PRB binding sites. CEAS: Enrichment on chromosome and annotation tool of Cistrome was used to analyze the distribution of binding sites with the following parameters: promoter/downstream -lower interval: 2500, -middle interval: 5000, - upper interval: 1000, bi-promoter -lower range: 5000, -upper range: 10000, relative distance: 5000.
- D. UCSC Genome Browser illustration of the localization of PRB binding site in *PGR* gene.
- E. ChIP-RT-qPCR validation of PRB binding in the first exon of *PGR* gene. ChIP was performed as described in materials and methods section. Chromatin enrichment was quantified by RT- PCR using primers flanking PRB binding site in the PRA promoter region. Negative region was a PRE deficient region. To calculate the enrichment of chromatin, the resulting signals were normalized to 1% input DNA. Data are represented as fold enrichment of PRA or PRB binding over that of the negative control ChIP. Data is representative of three independent experiments.
- F. HESC were transduced with increasing MOIs of adenovirus harboring PRA or PRB for 24h, control sample was not transduced. Next, cells were treated with differentiation cocktail for 6h. Whole cell protein extracts were prepared from these cells and analyzed by western blotting using a monoclonal antibody that recognizes both PRA and PRB (kindly provided by Dr. Dean Edwards of Baylor College of Medicine). Immunostaining of calnexin was used as a loading control.

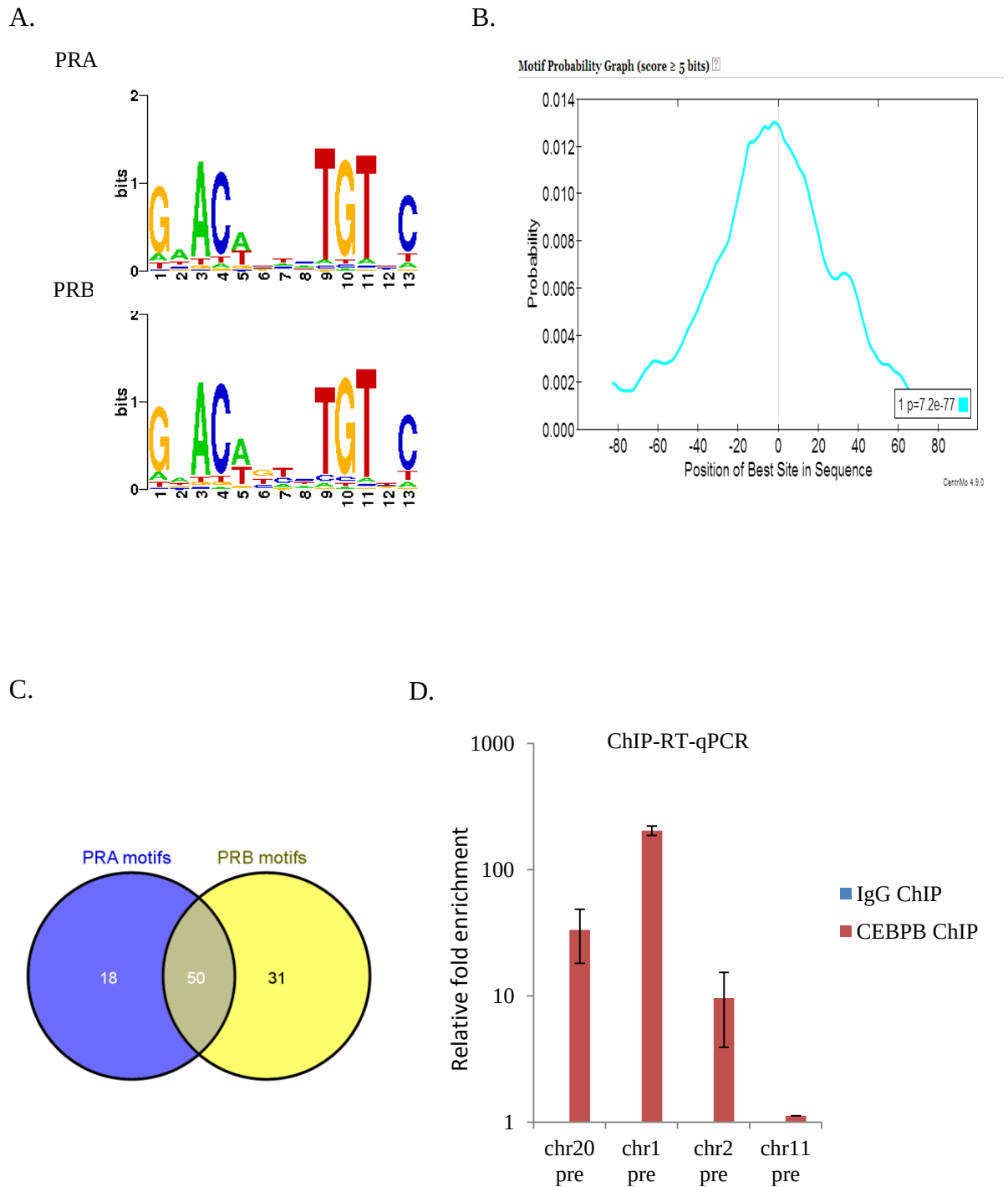


Figure 3.3: Motif analysis

A. PRE progesterone receptor binding motif for PRA and PRB identified by *de novo* motif analysis using Seqpos tool of Cistrome [22].

Figure 3.3 cont.:

B. Relative position of PRE within the summits of the binding sites was identified using MEME-ChIP [21].

C. Comparison of identified motifs within the 200nt window of PRA PRB binding sites.

HESC were subjected to treatment with differentiation cocktail for 48h. CEBPB ChIP was performed as described in material and methods. Chromatin enrichment was quantified by real-time PCR using primers flanking the CEBPB motif containing PRA and PRB binding sites (chr20 pre, chr1 pre, chr2 pre) and CEBPB motif negative PRB binding site (chr11 pre) as indicated in the figure. To calculate the enrichment of chromatin, the resulting signals were normalized to 1% input DNA. Data are represented as fold enrichment of CEBPB binding over that of the mock IgG ChIP. Graphs represent the mean $\pm$ SD of three independent experiments.

Table 3.1: List of motifs observed in the 200nt sequence around the summits of PRA binding sites

	factors	DNA binding domain	consensus	hits	zscore	- 10*log(p val)
1	<i>de novo</i> **	None	2AC4TGT2	3686	-49.89	1.00E-30
2	NR3C1 (GR)**	Hormone-nuclear Receptor Family	1G1AC5GT4	3626	-48.89	1.00E-30
3	<i>de novo</i> **	None	5ACA4GT2	3856	-48.67	1.00E-30
4	PGR**	Hormone-nuclear Receptor Family	G1AC5GT2	2919	-48.63	1.00E-30
5	AR**	Hormone-nuclear Receptor Family	3AC5GT1C2	3730	-47.59	1.00E-30
6	<i>de novo</i> **	None	4ACA4GT6	3196	-46.37	1.00E-30
7	<i>de novo</i> **	None	GAACAKWMTG	2857	-37.72	1.00E-30
8	<i>de novo</i> *	None	MAGAACA	3696	-27.18	1.00E-30
9	<i>de novo</i> *	None	CAGAACATTY	4020	-23.99	1.00E-30
10	<i>de novo</i> *	None	RVMMAGAACA	2598	-23.71	1.00E-30
11	<i>de novo</i> *	None	CTGTSCT	3810	-20.78	1.00E-30
12	Rest	BetaBetaAlpha-zinc finger Family	3A2AC3G3A3	243	-17.03	1.00E-30
13	<i>de novo</i>	None	CCAWGGACAG	375	-15.84	1.00E-30
14	<i>de novo</i> *	None	TSTGTTY	2482	-13.24	1.00E-30
15	<i>de novo</i>	None	AARSACA	3109	-13.16	1.00E-30
16	Snai1	BetaBetaAlpha-zinc finger Family	6CTG1CC1	186	-10.42	9.88E-26
17	<i>de novo</i> *	None	YTCTGTSCTB	3729	-10.18	1.21E-24
18	<i>de novo</i>	None	MRGAATG	2510	-8.90	2.72E-19
19	<i>de novo</i> *	None	T1TGT2T5	4397	-8.71	1.51E-18
20	Jun	Leucine zipper Family (bZIP)	ATGASTCAG	1844	-8.52	7.65E-18
21	NR1H4	Hormone-nuclear Receptor Family	AGGWCAT	2709	-8.48	1.15E-17
22	Gm5454	Ets Domain Family	4C2GA1GT4	3213	-8.42	1.86E-17
23	ZBTB12	BetaBetaAlpha-zinc finger Family	6T1GAAC5	3985	-8.37	2.93E-17
24	Esr1	Hormone-nuclear Receptor Family	5CA3TG1C2	4269	-7.99	6.80E-16
25	Etv5	Ets Domain Family	4C1GGA1G5	4017	-7.93	1.11E-15
26	Elk3	Ets Domain Family	5C1GGAA6	4146	-7.68	7.70E-15
27	ZNF205	BetaBetaAlpha-zinc finger Family	SARAATG	1527	-7.61	1.33E-14
28	NR4A1	Hormone-nuclear Receptor Family	YTGWCCTTKB	4511	-7.25	2.07E-13
29	ESR2	Hormone-nuclear Receptor Family	9CA3TG1C2	4914	-7.17	3.69E-13
30	Nkx3-1	Homeodomain Family	2TRTKT6	3726	-7.14	4.67E-13
31	NR4A2	Hormone-nuclear Receptor Family	AAGRWCAGV	4911	-7.09	6.71E-13
32	NR2C2	Hormone-nuclear Receptor Family	SARAGKWCAG R	4596	-6.79	5.66E-12
33	ELK4	Ets Domain Family	4C1GGAA6	3838	-6.70	1.05E-11
34	HSF1	Transcription Factor Family	MTGGAACA	3421	-6.69	1.15E-11
35	FOSL2	Leucine zipper Family (bZIP)	TGACTCA	2416	-6.65	1.46E-11

36	JUND	Leucine zipper Family (bZIP)	TGAGTCAY	2486	-6.46	5.16E-11
37	SOX10	High Mobility Group (Box) Family	ACAMWG	3007	-6.21	2.70E-10
38	JUNB	Leucine zipper Family (bZIP)	MTGASTCAYM	2144	-5.83	2.77E-09
39	Zfp691	BetaBetaAlpha-zinc finger Family	5AGT1CT6	4301	-5.58	1.20E-08
40	NFE2	Leucine zipper Family (bZIP)	WRMTGASTCAY	1902	-5.54	1.48E-08
41	BACH2	Leucine zipper Family (bZIP)	VRTGAGTCATM	2512	-5.48	2.07E-08
42	HIF1A	Helix-Loop-Helix Family (bHLH)	MDKCACGTHY	3079	-5.37	3.93E-08
43	BACH1	Leucine zipper Family (bZIP)	4T1AGT1A4	1446	-5.34	4.59E-08
44	NKX2-3	Homeodomain Family	4AAG2CT5	2905	-5.32	5.13E-08
45	FOSL1	Leucine zipper Family (bZIP)	RTGASTCABM	2277	-5.30	5.66E-08
46	Tbx21	Transcription Factor T-Domain	RTGASTCAKM	2351	-5.16	1.22E-07
47	Nr5a2	Hormone-nuclear Receptor Family	TGDCCTTGRR	2508	-5.14	1.38E-07
48	Jund	Leucine zipper Family (bZIP)	8TGA1T1A1	1611	-5.06	2.06E-07

**similar to PRE (progesterone response element)

*similar to half PRE

Table 3.1 cont.:

De novo motif analysis was performed on 200nt sequences around summits of top 5000 PRA binding sites using SeqPos tool of Cistrome. Motifs are sorted by z-score with a cut off value of -5.

Table 3.2: List of motifs observed in the 200nt sequence around the summits of PRB binding sites

	factors	DNA binding domain	consensus	hits	zscore	- 10*log(p val)
1	<i>de novo</i> **	None	2AC4TGT2	4824	-67.52	1.00E-30
2	<i>de novo</i> **	None	2G1AC5GT3	4827	-66.67	1.00E-30
3	NR3C1 (GR)**	Hormone-nuclear Receptor Family	1G1AC5GT4	4313	-65.81	1.00E-30
4	AR**	Hormone-nuclear Receptor Family	3AC5GT1C2	4535	-64.33	1.00E-30
5	PGR**	Hormone-nuclear Receptor Family	G1AC5GT2	4671	-64.23	1.00E-30
6	<i>de novo</i> **	None	3ACA5T1C5	4600	-56.38	1.00E-30
7	<i>de novo</i> *	None	CAKWMGTGTTTC	4120	-50.47	1.00E-30
8	<i>de novo</i> *	None	3CTGT1C4	4746	-40.16	1.00E-30
9	<i>de novo</i> *	None	MTGTTCY	3387	-39.00	1.00E-30
10	<i>de novo</i> *	None	MTGTYCT	4934	-38.70	1.00E-30
11	<i>de novo</i> *	None	TGTTCTG	3695	-36.42	1.00E-30
12	<i>de novo</i> *	None	WMTGTYCTKK	3611	-36.07	1.00E-30
13	<i>de novo</i> *	None	TGTTCTKKKY	2777	-35.11	1.00E-30
14	<i>de novo</i> *	None	T2TGT1C8	4547	-26.93	1.00E-30
15	<i>de novo</i>	None	WRRAMWGTY	4965	-24.90	1.00E-30
16	<i>de novo</i> *	None	TGT1CT7	4237	-23.33	1.00E-30
17	<i>de novo</i> *	None	TGTVYTT	3650	-20.33	1.00E-30
18	<i>de novo</i>	None	GAATGTR	2810	-17.76	1.00E-30
19	<i>de novo</i>	None	CAGRAWG	4634	-16.81	1.00E-30
20	<i>de novo</i>	None	CATBCTSTCC	3471	-16.20	1.00E-30
21	<i>de novo</i>	None	TMCAGAA	4933	-15.86	1.00E-30
22	<i>de novo</i>	None	SMCAGRA	4839	-14.58	1.00E-30
23	Elk3	Ets Domain Family	6TTCC1G5	4943	-13.46	1.00E-30
24	NR1H4	Hormone-nuclear Receptor Family	AGGWCAT	3160	-13.42	1.00E-30
25	Gm5454	Ets Domain Family	4AC1TC2G4	4628	-13.39	1.00E-30
26	Etv5	Ets Domain Family	5C1TCC1G4	4524	-13.35	1.00E-30
27	ESR2	Hormone-nuclear Receptor Family	9CA3TG1C2	4960	-12.77	1.00E-30
28	ZBTB12	BetaBetaAlpha-zinc finger Family	5C2GAAC5	4151	-12.73	1.00E-30
29	Esr1	Hormone-nuclear Receptor Family	5CA3TG1C2	4320	-12.28	1.00E-30
30	ELK4	Ets Domain Family	6TTCC1G4	4726	-12.09	1.00E-30
31	HSF1	Transcription Factor Family	MTGGAACA	4428	-11.78	1.00E-30
32	Rest	BetaBetaAlpha-zinc finger Family	3T1T1C4T2T3	127	-11.71	1.00E-30
33	ZNF205	BetaBetaAlpha-zinc finger Family	SARAATG	1587	-11.69	1.00E-30
35	HSF2	Transcription Factor Family	TT5A2TT1	1941	-11.23	1.50E-29

Table 3.2 cont.

36	PAX6	Homeodomain Family	3T3AG2CA1	3115	-10.97	2.72E-28
38	NR2C2	Hormone-nuclear Receptor Family	SARAGKWCWG D	4418	-10.53	3.28E-26
39	REST	BetaBetaAlpha-zinc finger Family	7CT1TCC3	212	-10.12	2.23E-24
41	NR4A2	Hormone-nuclear Receptor Family	BTGWYCTT	4979	-10.05	4.42E-24
42	Hsf1	Transcription Factor Family	6C2G4TTC2	2693	-9.78	6.81E-23
43	Spdef	Ets Domain Family	5TCCKG6	4964	-9.54	7.48E-22
44	SOX10	High Mobility Group (Box) Family	CWKTGT	4962	-9.46	1.50E-21
45	PAX1	Homeodomain Family	CWGGAACWM AM	4955	-9.27	9.71E-21
46	ETV4	Ets Domain Family	4A2TCC1G4	4947	-8.78	7.92E-19
47	ETV1	Ets Domain Family	ACAGGAART	406	-8.27	6.79E-17
48	Tead4	Homeodomain Family	6GGAAT2	864	-8.02	5.16E-16
49	MTF1	BetaBetaAlpha-zinc finger Family	1T4A4TTT6	2790	-7.72	5.77E-15
50	HSF1	Transcription Factor Family	2C2G1A3TC	4706	-7.57	1.86E-14
51	Trp63	Loop-Sheet-Helix Family (bHSH)	6G5AC1TG3	4139	-7.52	2.67E-14
52	Fli1	Ets Domain Family	ACAGGAART	441	-7.41	6.54E-14
53	Hnf4a	Hormone-nuclear Receptor Family	4CT1TG2C4	4212	-7.21	2.76E-13
54	IKZF1	BetaBetaAlpha-zinc finger Family	3RTTCC5	4392	-7.17	3.74E-13
55	EPAS1	Leucine zipper Family (bZIP)	RWACGTGMKY	4986	-7.16	4.03E-13
56	HIF1A	Helix-Loop-Helix Family (bHLH)	RDACGTGMHK	3183	-7.15	4.38E-13
57	ELF2	Ets Domain Family	6TTCC1G4	4045	-7.15	4.41E-13
59	NKX2-3	Homeodomain Family	5AG2CTT4	653	-6.78	5.86E-12
60	Zfp187	BetaBetaAlpha-zinc finger Family	3TT4CAT2	2195	-6.50	4.07E-11
61	Zfp691	BetaBetaAlpha-zinc finger Family	6AG1ACT5	4989	-6.49	4.36E-11
62	<i>de novo</i>	None	CWGGGRAG	4167	-6.47	4.79E-11
63	Zfp128	BetaBetaAlpha-zinc finger Family	5K1C1TAC5	4130	-6.28	1.67E-10
64	Ikzf3	BetaBetaAlpha-zinc finger Family	YWYTTCCTSY	4842	-6.19	3.02E-10
65	SOX13	High Mobility Group (Box) Family	5AACAA6	4757	-6.11	5.04E-10
66	Stat5b	Stat Protein Family	TTCYARGAAA	2509	-6.06	6.93E-10
67	ESR1	Hormone-nuclear Receptor Family	1K2C4TG1C3	4988	-5.92	1.62E-09
68	Ets1	Ets Domain Family	3C1TCCT4	4147	-5.84	2.60E-09
69	Snai1	BetaBetaAlpha-zinc finger Family	6CTGT1C1	4228	-5.75	4.36E-09
70	TEF	Leucine zipper Family (bZIP)	VWSWGAATG TR	2381	-5.72	5.33E-09
71	TEAD1	Homeodomain Family	VWSWGAATG TR	2381	-5.64	8.61E-09
72	MTF1	BetaBetaAlpha-zinc finger Family	4T1CACA6	4985	-5.63	9.23E-09
73	Elf4	Ets Domain Family	6TTCC1G4	4945	-5.50	1.89E-08
74	CEBPB	Leucine zipper Family (bZIP)	1TK3CAA4	479	-5.37	3.98E-08

75	Cebpa	Leucine zipper Family (bZIP)	4TT1C2MA1	906	-5.28	6.49E-08
76	Irx3	Homeodomain Family	5ACATG7	4988	-5.23	8.46E-08
77	SOX5	High Mobility Group (Box) Family	5AACAA6	2924	-5.15	1.33E-07
78	NR4A1	Hormone-nuclear Receptor Family	YTGWCCTTKB	935	-5.07	1.97E-07
79	CEBPA	Leucine zipper Family (bZIP)	3TT3CAA2	621	-5.07	2.03E-07
80	CEBPE	Leucine zipper Family (bZIP)	RTKWYRCAAY	829	-5.06	2.08E-07

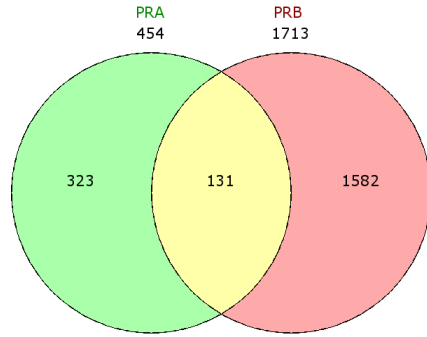
**similar to PRE (progesterone response element)

*similar to half PRE

Table 3.2 cont.:

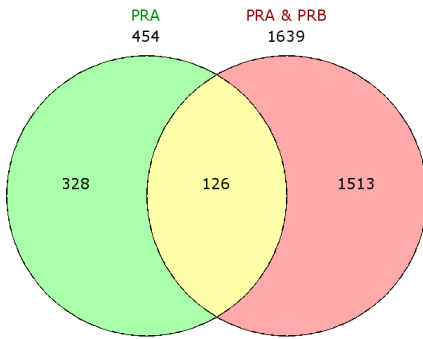
De novo motif analysis was performed on 200nt sequences around summits of top 5000 PRB binding sites using SeqPos tool of Cistrome. Motifs are sorted by z-score with a cut off value of -5.

A.



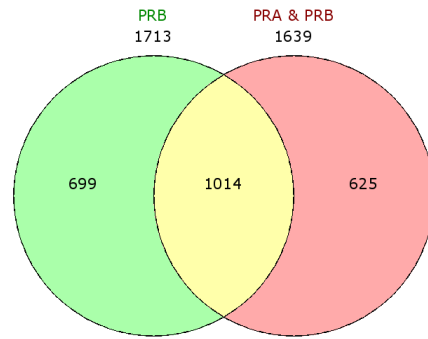
Lists contain 2036 unique elements

B.



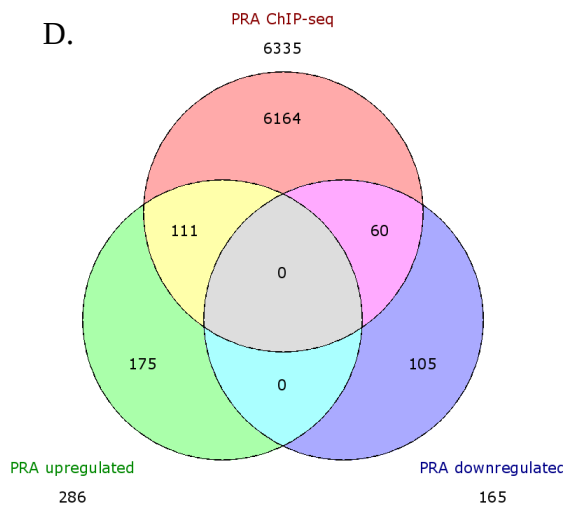
Lists contain 1967 unique elements

C.



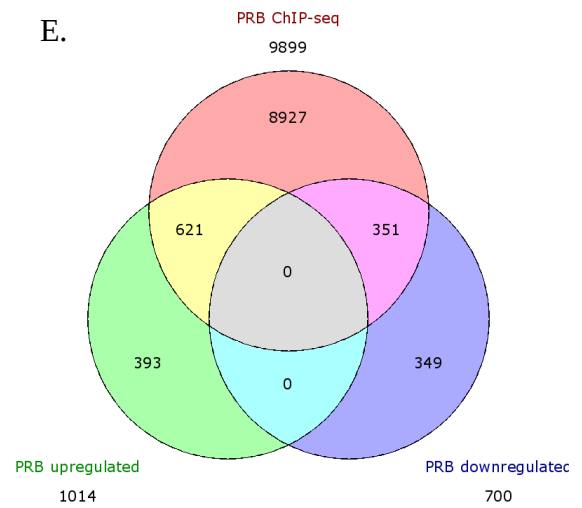
Lists contain 2338 unique elements

D.



Lists contain 6615 unique elements

E.



Lists contain 10641 unique elements

Figure 3.4: Gene networks downstream of PRA, PRB, or PRA & PRB co-expressing HESC

Figure 3.4 cont.:

- A. Venn diagram represents the number and overlap of the PRA regulated genes with that of PRB regulated genes.
- B. Venn diagram represents the number and overlap of the PRA regulated genes with that of PRA-PRB co-regulated genes
- C. Venn diagram represents the number and overlap of the PRB regulated genes with that of PRA-PRB co-regulated genes
- D. Venn diagram represents the number and overlap of the PRA upregulated and downregulated genes with list of genes with PRA binding sites within a window of $\pm 100\text{kb}$ from their TSS.
- E. Venn diagram represents the number and overlap of the PRB upregulated and downregulated genes with list of genes with PRB binding sites within a window of $\pm 100\text{kb}$ from their TSS.

Table 3.3: Functional Annotation Clustering of PRA regulated genes

Functional annotation clustering of PRA upregulated genes						
Cluster	Enrichment Score	Category	Representative Term	Count	%	PValue
1	6.82	GOTERM_BP_FAT	Regulation of apoptosis	43	14.63	1.26E-07
2	5.76	GOTERM_BP_FAT	Negative regulation of apoptosis	25	8.50	1.47E-06
3	3.67	GOTERM_BP_FAT	Protein amino acid phosphorylation	31	10.54	5.88E-05
4	3.41	GOTERM_BP_FAT	Regulation of angiogenesis	17	5.78	1.83E-04
5	3.17	GOTERM_BP_FAT	Cell death	31	10.54	3.66E-04
6	3.12	GOTERM_BP_FAT	Positive regulation of apoptosis	22	7.48	7.08E-04
7	3.10	GOTERM_BP_FAT	Regulation of cell motion	14	4.76	5.43E-04
8	3.08	UP_SEQ_FEATURE	Protein kinase	22	7.48	2.34E-04
9	2.92	GOTERM_BP_FAT	Positive regulation of cell motion	10	3.40	5.47E-04
10	2.89	GOTERM_BP_FAT	Branching morphogenesis of a tube	8	2.72	7.96E-04
11	2.84	GOTERM_BP_FAT	Regulation of adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	8	2.72	2.08E-04

Functional annotation clustering of PRA downregulated genes						
Cluster	Enrichment Score	Category	Representative Term	Count	%	PValue
1	2.90	SP_PIR_KEYWORDS	Transcription	37	20.33	4.87E-04
2	2.06	GOTERM_MF_FAT	Translation elongation factor activity	4	2.20	0.002774801
3	1.55	GOTERM_BP_FAT	Cellular cation homeostasis	10	5.49	0.008326236

Table 3.3 cont.:

The DAVID gene functional annotation tool was used for the gene-GO term enrichment analysis of PRA regulated genes. The upregulated and downregulated genes were classified separately into the different GO FAT terms (Biological Process) with high classification stringency.

Table 3.4: Functional Annotation Clustering of PRB regulated genes

Functional annotation clustering of PRB upregulated genes						
Cluster	Enrichment Score	Category	Representative Term	Count	%	PValue
1	10.39	GOTERM_CC_FAT	Lysosomal proteins	44	4.28	2.35E-10
2	6.89	UP_SEQ_FEATURE	Transmembrane region proteins	324	31.49	2.97E-09
3	6.23	GOTERM_BP_FAT	Regulation of angiogenesis	44	4.28	3.41E-07
4	6.09	GOTERM_BP_FAT	Response to extracellular stimulus	38	3.69	1.54E-06
5	5.71	GOTERM_BP_FAT	Regulation of cell motion	36	3.50	1.02E-06
6	5.11	SMART	Histone core H2B	8	0.78	5.76E-06
7	4.66	GOTERM_BP_FAT	Regulation of apoptosis	92	8.94	1.58E-05
8	4.25	GOTERM_BP_FAT	Nucleosome assembly	17	1.65	1.92E-05
9	4.09	GOTERM_BP_FAT	Cell death	79	7.68	7.45E-05
10	3.74	GOTERM_BP_FAT	Positive regulation of cell migration	19	1.85	1.76E-04
11	3.68	SP_PIR_KEYWORDS	Egf-like domain	31	3.01	1.49E-04
12	3.67	GOTERM_MF_FAT	Insulin-like growth factor binding	10	0.97	2.48E-05
13	2.86	GOTERM_BP_FAT	Regulation of cell migration	37	3.60	5.42E-04
14	2.86	GOTERM_BP_FAT	Positive regulation of cell death	51	4.96	0.001069
15	2.60	GOTERM_BP_FAT	Regulation of phosphate metabolic process	53	5.15	0.002019

Functional annotation clustering of PRB downregulated genes						
Cluster	Enrichment Score	Category	Representative Term	Count	%	PValue
1	56.30	GOTERM_BP_FAT	Cell cycle phase	120	16.51	6.28E-62
2	51.02	GOTERM_BP_FAT	M phase	106	14.58	1.81E-61
3	21.65	GOTERM_CC_FAT	Kinetochore	32	4.40	5.09E-22
4	17.83	GOTERM_CC_FAT	Nuclear lumen	152	20.91	2.59E-22
5	16.96	GOTERM_CC_FAT	Microtubule cytoskeleton	88	12.10	4.25E-27
6	10.11	GOTERM_BP_FAT	M phase of meiotic cell cycle	23	3.16	6.36E-11
7	10.06	GOTERM_BP_FAT	DNA repair	46	6.33	1.52E-12

Table 3.4 cont.:

The DAVID gene functional annotation tool was used for the gene-GO term enrichment analysis of PRB regulated genes. The upregulated and downregulated genes were classified separately into the different GO FAT terms (Biological Process) with high classification stringency.

Table 3.5: Functional Annotation Clustering of PRA+PRB regulated genes

Functional annotation clustering of PRA+PRB upregulated genes						
Cluster	Enrichment Score	Category	Representative Term	Count	%	PValue
1	9.03	GOTERM_CC_FAT	Lysosomal proteins	41	4.13	3.07E-09
2	6.62	GOTERM_CC_FAT	Transmembrane region proteins	339	34.17	3.83E-06
3	4.06	INTERPRO	Histone core H2B	7	0.71	8.15E-05
4	3.97	GOTERM_BP_FAT	Regulation of cell migration	29	2.92	4.94E-05
5	3.76	GOTERM_MF_FAT	Insulin-like growth factor binding	10	1.01	2.16E-05
6	3.41	GOTERM_CC_FAT	Nucleosome	15	1.51	1.56E-05
7	3.28	GOTERM_MF_FAT	Endopeptidase activity	42	4.23	1.61E-04
8	3.17	GOTERM_BP_FAT	Cell death	74	7.46	4.23E-04
9	2.92	GOTERM_BP_FAT	Response to nutrient levels	29	2.92	4.30E-04
10	2.22	KEGG_PATHWAY	Chondroitin sulfate biosynthesis	8	0.81	5.78E-04
11	2.22	GOTERM_BP_FAT	Positive regulation of phosphate metabolic process	16	1.61	0.003487
12	2.18	GOTERM_BP_FAT	Regulation of apoptosis	77	7.76	0.006042
13	2.11	GOTERM_MF_FAT	Serine-type endopeptidase activity	19	1.92	0.00404
14	2.03	GOTERM_BP_FAT	Aminoglycan metabolic process	11	1.11	0.004328

Functional annotation clustering of PRA+PRB downregulated genes						
Cluster	Enrichment Score	Category	Representative Term	Count	%	PValue
1	55.58	GOTERM_BP_FAT	Cell cycle phase	112	16.77	3.08E-58
2	46.37	GOTERM_BP_FAT	M phase	98	14.67	1.26E-56
3	40.40	SP_PIR_KEYWORDS	Cell division	75	11.23	3.38E-42
4	22.44	GOTERM_CC_FAT	Nuclear lumen	155	23.20	9.90E-28
5	21.99	GOTERM_CC_FAT	Kinetochore	32	4.79	4.86E-23
6	16.96	GOTERM_CC_FAT	Microtubule cytoskeleton	85	12.72	1.70E-27
7	11.78	GOTERM_BP_FAT	M phase of meiotic cell cycle	24	3.59	1.34E-12
8	8.85	UP_SEQ_FEATURE	Nucleotide phosphate-binding region:ATP	88	13.17	3.63E-12

Table 3.5 cont.:

The DAVID gene functional annotation tool was used for the gene-GO term enrichment analysis of differentially regulated genes in PRA+PRB co-expressing cells. The upregulated and downregulated genes were classified separately into the different GO FAT terms (Biological Process) with high classification stringency.

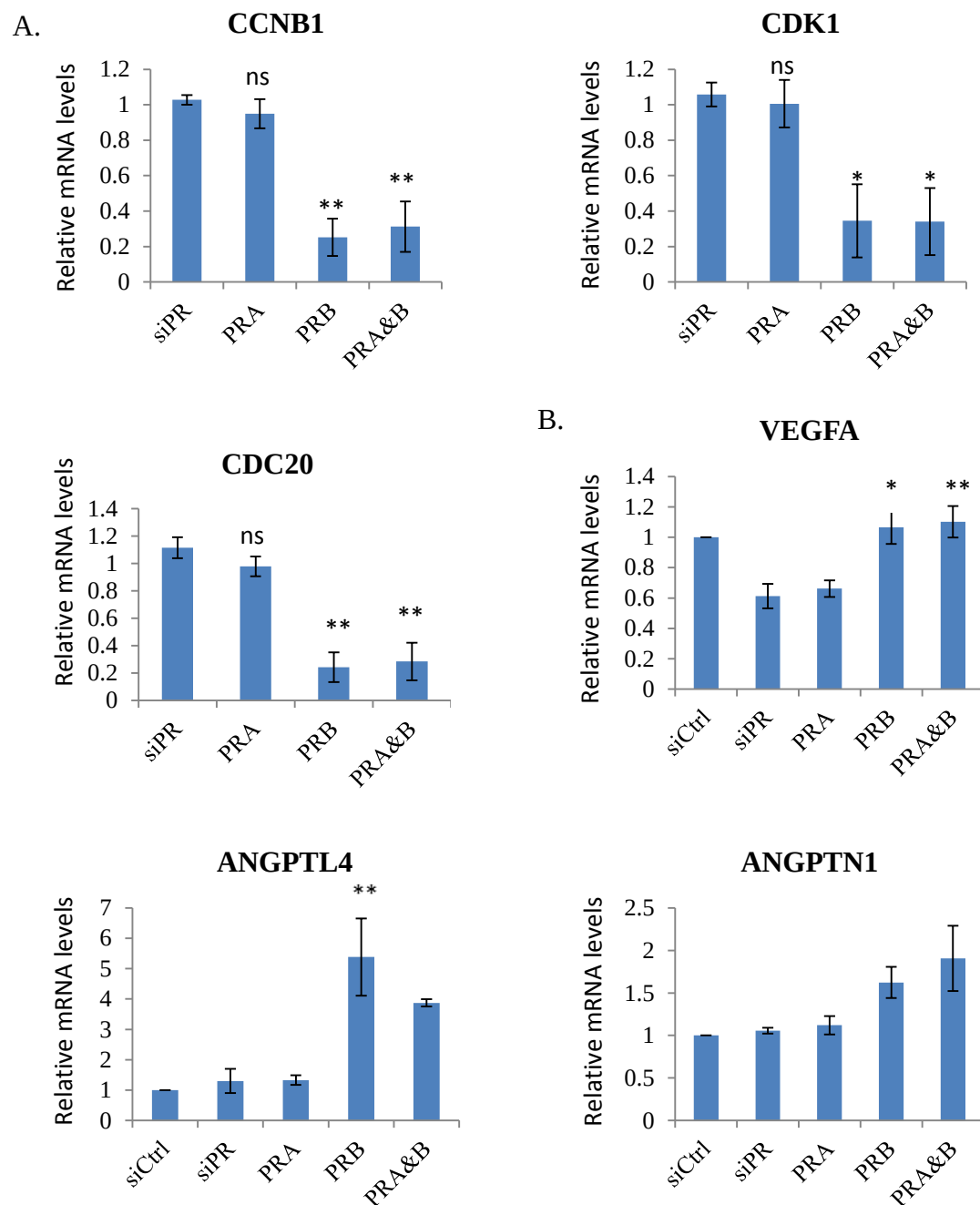


Figure 3.5: Validation of PRB regulation of cell cycle and angiogenesis related molecules

Figure 3.5 cont.:

- A. HESC were treated with 50nM control siRNA or siRNA targeting 3'UTR of PGR mRNA. Next, cells were transduced with adenovirus harboring flag-tagged PRA or PRB or control virus for 24h. Then, siRNA and adenovirus were removed and cells were treated with differentiation cocktail. Total RNA was isolated at 6h and cDNAs were prepared. cDNAs were subjected to real-time PCR using gene specific primers for CCNA2, CCNB1, CDC20, CDC25A, CDK1 and 36B4. PCR data are normalized to 36B4 mRNA levels. Relative fold changes were normalized with respect to control siRNA treatment. Graphs represent the mean $\pm$ SEM of three independent experiments. One-way ANOVA was used for statistical analysis; $p \leq 0.05^*$, $p \leq 0.01^{**}$.
- B. Same samples were used to run real time PCR using gene specific primers for ANGPTN1, ANGPTNL4 and VEGFA. Graphs represent the mean $\pm$ SEM of three independent experiments. One-way ANOVA test was used for statistical analysis; $p \leq 0.05^*$, $p \leq 0.01^{**}$.

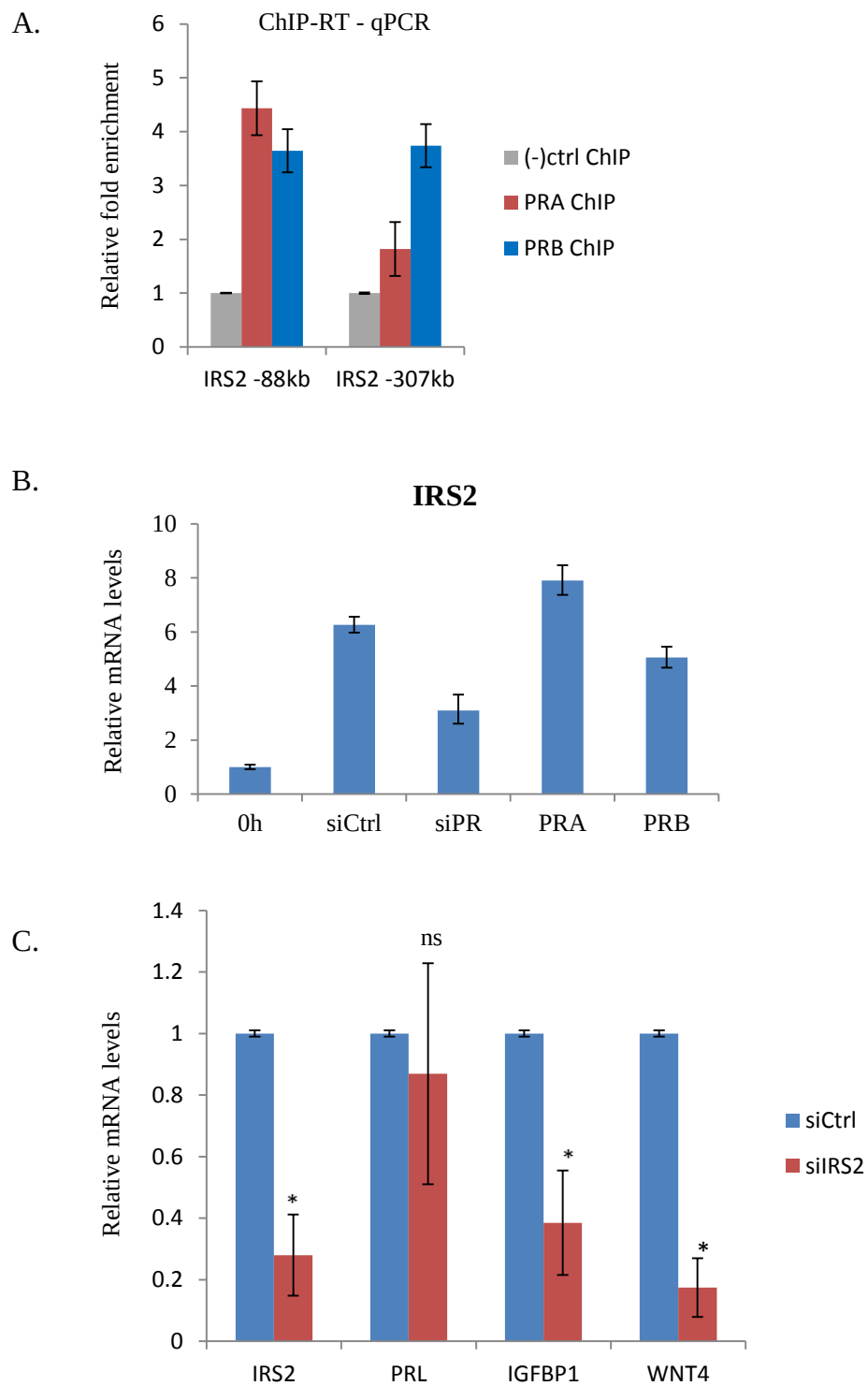


Figure 3.6: IRS2 is regulated by PRA and PRB and critical for stromal differentiation

Figure 3.6 cont.:

- A. HESC were transduced with or without recombinant adenovirus harboring flag-tagged PRA or PRB for 24 h. Then, samples were subjected to treatment with differentiation cocktail for 2 h. ChIP was performed as described in material and methods. Chromatin enrichment was quantified by real-time PCR using primers flanking potential PRA and PRB binding sites in the 5'-flanking region of *IRS2* at -88kb and -307kb. The experiment was repeated three times and representative data are shown.
- B. HESC were treated with 50nM control siRNA or siRNA targeting 3'UTR of PGR mRNA. Next, cells were transduced with adenovirus harboring flag-tagged PRA or PRB or control virus for 24h. Then, siRNA and adenovirus were removed and cells were treated with differentiation cocktail. Total RNA was isolated at 24h after differentiation cocktail addition and cDNAs were prepared. cDNAs were subjected to real-time PCR using gene specific primers for *IRS2*. PCR data are normalized to 36B4 mRNA levels. Relative fold changes were normalized with respect to basal levels of *IRS2* before differentiation cocktail addition indicated as 0h in the figure. Experiment was performed two times and representative data are shown.
- C. HESCs were transfected with siRNA targeting *IRS2* or scrambled siRNA (control). 48h after transfection, HESCs were treated with differentiation cocktail to initiate *in vitro* differentiation. Cells were harvested at 72h following differentiation cocktail treatment. Total RNA was isolated and cDNAs were prepared. cDNAs were subjected to real-time PCR using gene specific primers for *IRS2*, *PRL*, *IGFBP1* and *WNT4*. PCR data are normalized to 36B4 mRNA levels. Relative fold changes were normalized with respect to control non-targeting siRNA treatment. Graphs represent the mean $\pm$ SD of three independent experiments ($p \leq 0.05^*$).

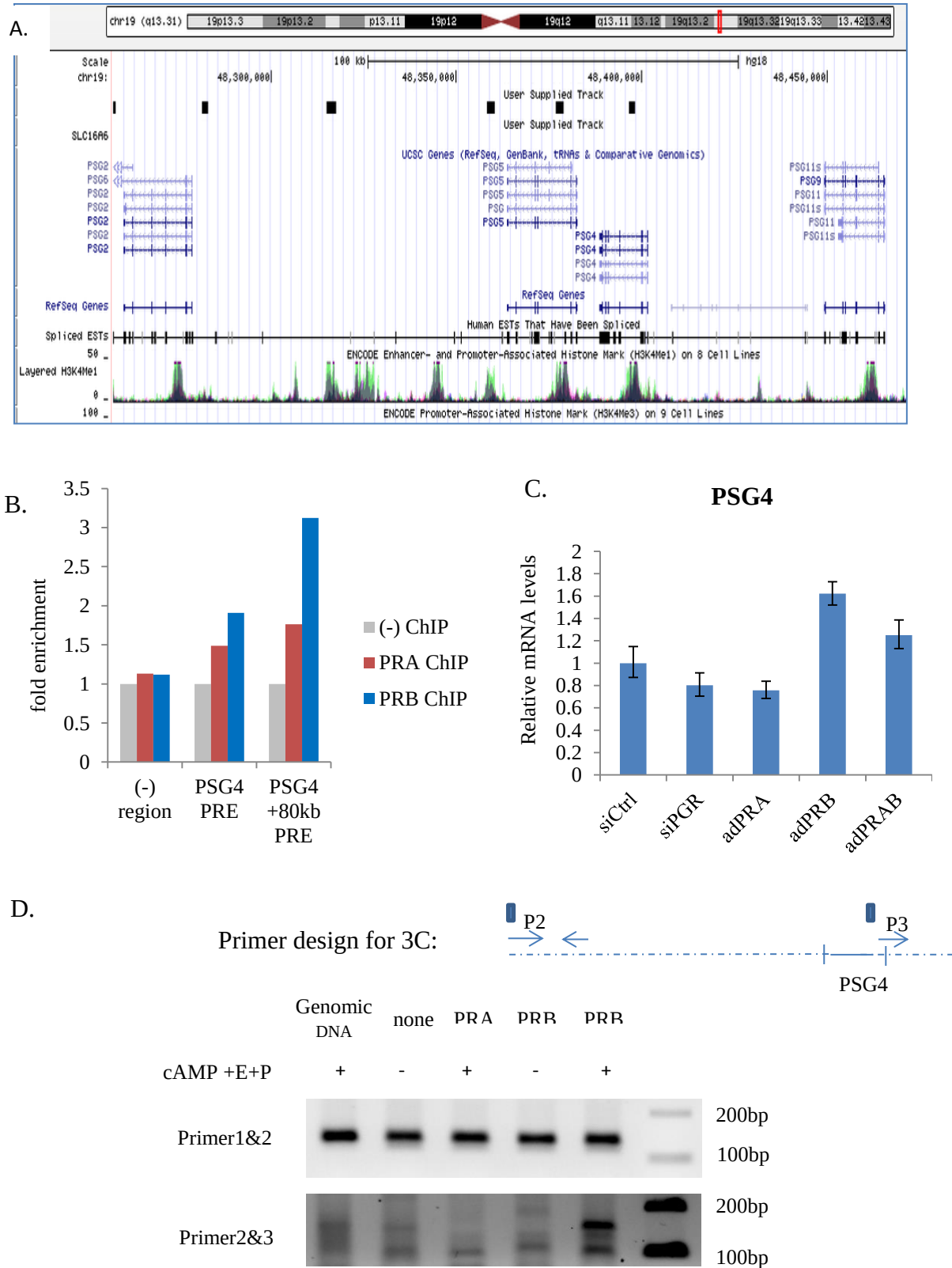


Fig. 3.7: Interaction of a distal and a proximal progesterone receptor binding site of a PRB target gene, PSG4

Fig. 3.7 cont.:

Chromosome conformation capture assay (3C) was performed using HESC were untreated or transduced with adenovirus harboring PRA and PRB cDNA. At 24h, cells were untreated or treated with differentiation cocktail for 30 min as indicated in the figure. 3C was performed as explained elsewhere [54]. **A.**UCSC genome browser snapshot showing the locations of PRB binding sites scattered around PSG genes. **B.** ChIP-qPCR validation of PRB binding in the intron and enhancer-like upstream region of *PSG4* gene. ChIP was performed as described in materials and methods section. Chromatin enrichment was quantified by RT-PCR. Negative region was a PRE deficient region. To calculate the enrichment of chromatin, the resulting signals were normalized to 1% input DNA. Data are represented as fold enrichment of PRA or PRB binding over that of the negative control ChIP. Data is representative of two independent experiments. **C.** Upper panel: Illustration of PRB binding sites and localization of 3C primers. Lower panel: 3C PCR results. This experiment was performed once. Primer pair 1&2 were designed to amplify a genomic region free of restriction enzyme digestion sites as a loading control between the samples. Primer pair 2&3 would amplify a product (~140bp) as a result of an intermolecular ligation of the proximal and distal PRB binding sites.

CHAPTER IV

CONCLUSIONS AND FUTURE PERSPECTIVES

ESR1 plays a critical role in human decidualization

To improve our understanding of the molecular mechanisms controlling uterine decidualization and better address the underlying causes of various endometrial disorders, it is important to extend the information gained from the mouse models to the clinical realm. In our laboratory, we developed several conditional knockout mouse models to address the functional roles of a variety of maternal factors, such as transcription factors, cytokines, morphogens, etc., during early pregnancy. A major focus of our laboratory is to use primary cultures of human endometrial cells to examine in parallel the roles of these molecules in the human.

One of the main objectives of my project was to address the role of estrogen receptor alpha during endometrial stromal differentiation in the human. Previously, our laboratory developed conditional knockout mice in which the “floxed” *Esr1* gene was deleted in the uterine epithelial and stromal cells by a progesterone receptor (PR)-driven Cre. This genetically altered mouse model showed that the expression of estrogen receptor alpha (ESR1) in the uterus is essential for proper differentiation of the endometrium (Mary J. Laws, Milan K. Bagchi and Indrani C. Bagchi, unpublished data). However, since *Esr1* was deleted in both epithelial and stromal cells, it was not possible to distinguish between the stromal versus epithelial functions of ESR1 in decidualization. In the present study, we used primary human endometrial stromal cells to examine the role of ESR1 in human uterine differentiation. Silencing of *ESR1* expression by RNA interference increased stromal cell proliferation and inhibited differentiation. We showed that ESR1 suppressed a number of key cell cycle regulatory molecules so that the stromal cells could cease to proliferate and were able to enter the differentiation program. There were many similarities in downstream gene networks regulated by ESR1 in the mouse and the human. These included factors involved in hepatocyte growth factor signaling, growth hormone signaling, polo-like kinase signaling and regulation of the cell cycle. Down regulation of *ESR1* expression

in human endometrial stromal cells impaired the expression of *PR*, *WNT4*, and *FOXO1*, which are known regulators of the stromal differentiation process in mice. Our studies, therefore, established a critical and conserved role of ESR1 during decidualization in the human.

We also observed that, during stromal differentiation, ESR1 acts in concert with cAMP signaling to modulate target gene expression. Our studies revealed that the mediator complex (MED1), a critical transcription co-regulatory factor, is modified by phosphorylation by cAMP-dependent PKA and serves as a link between ESR1 and the basal transcription machinery to promote target gene transcription. We, however, do not rule out that additional factors may interact with ESR1 and modulate its transcriptional activity.

The finding that ESR1 suppresses the proliferation of endometrial stromal cell during decidualization was surprising. Estrogen generally induces cell proliferation in tissues, such as the uterine and mammary epithelia. However, there are scenarios where estrogen suppresses cell cycle progression. For instance, estrogen treatment induces proliferation of human MCF7 breast tumor cells while it represses proliferation of MDA-MB-231 tumor cells [1]. The difference in its proliferative response in different cell types may be due to inherent differences in the composition, levels, and activities of various signaling molecules, transcription factors and cofactors. This concept is supported by a previous report that the antiestrogen ICI 182780 has no inhibitory effect on proliferation of certain immortalized endometrial cells [2]. In fact, it exerts a stimulatory effect at low concentrations. Collectively, these findings suggest that targeting of ESR1 for alleviation of endometrial stromal tumors may be ineffective or even have an unfavorable consequence. A clear understanding of the mechanisms that control endometrial growth and differentiation is important for developing effective treatment strategies for endometrial diseases.

Role of PR isoforms in human decidualization

It is well established that P is the most important regulator of stromal differentiation in both mice and humans. Female mice lacking the PRA isoform are infertile and present with several reproductive abnormalities, including loss of uterine receptivity and impaired stromal decidualization [3-5]. On the other hand, PRB-null mice are fertile and retain normal decidualization response [6]. To address the specific roles of PRA and PRB in human

decidualization, we independently expressed PRA and PRB in human stromal cell cultures. Our findings, summarized in Chapter III, suggest that, unlike what was observed in mice, PRB is a more potent transcriptional regulator than PRA during human stromal cell differentiation. We determined that PRB interacted with and regulated many more target genes than PRA. We also found that when the two isoforms are co-expressed, the PRB activity is predominant.

Many human endometrial disorders, such as endometriosis and endometrial cancer, are associated with altered PRA:PRB ratio. For example, in ectopic endometriotic tissue, PRA, but no PRB protein, is detected [7]. This suppression of PRB expression in endometriosis was linked to the hypermethylation of its promoter [8]. Differential expression of PR isoforms was also reported in endometrial cancer. Whereas well-differentiated cancer cells (Ishikawa) expressed both PRA and PRB, the poorly differentiated cancer cells (HecSO and KLE) expressed only PRA [9]. One of the well-established roles of P signaling in the endometrium is suppression of cell proliferation [10]. Our study suggested that PRB inhibited proliferation of stromal cells by suppressing the expressions of key cell cycle regulators, including *CCNB1*, *CDK1* and *CDC20*, while PRA did not affect their expression. We believe that this finding is critical for understanding endometrial disorders, such as endometrial cancers and endometriosis, and reduced expression of PRB may be a critical predictive marker for cancers that are unresponsive to progestin therapy.

Identification of potential direct targets of PRA and PRB during human decidualization

Using ChIP-seq analysis, we identified a large number of genes as potential targets of regulation by PRA and PRB during decidualization of human endometrial stromal cells. Prominent among these genes is the transcription factor *HAND2* (heart- and neural crest derivatives-expressed protein 2), which was previously shown to be required for implantation and decidualization in the mouse [11]. Our laboratory is currently analyzing the functions of the PRA and PRB binding sites in regulation of *HAND2* expression during various phases of the implantation process. *BMP2* (bone morphogenetic protein 2) was also reported to be downstream of P and required for differentiation of stromal cells in both mice and human [12, 13]. We identified a PRB binding site in the regulatory region of this gene, suggesting that P regulation of *BMP2* might be via direct binding of this site by PRB. We also identified *HOXA10* and *HOXA11*

as potential direct targets of PRB. These transcription factors, which belong to the Hox family of genes, are required for successful pregnancy in mice [14, 15]. The P-regulated expression of *HOXA10* and *HOXA11* occurs in human endometrium within the window of implantation [16, 17]. We identified several other critical regulators of stromal differentiation, such as *KLF4*, *KLF9*, and *STAT3*, as potential direct targets of PRA and PRB. These findings provided an understanding of the conserved molecular pathways by which P, acting via its receptors, regulates decidualization in the mouse and the human. In addition, several other factors, identified as PR target genes in our study, may have as yet unknown functions during decidualization.

P regulation of energy metabolism during decidualization

Our studies uncovered an interesting link between PR signaling and regulation of energy metabolism during decidualization. Critical endometrial functions, such as proliferation, differentiation and apoptosis are energy demanding processes. Growing evidence suggests that the regulation of energy metabolism is essential for the establishment of successful pregnancy [18]. For example, an adequate level of glucose is necessary for proper stromal differentiation. During *in vitro* differentiation of mouse and human stromal cells, the differentiation process is impaired when the glucose levels in the culture medium are depleted [19]. Several facilitative glucose transporters (GLUTs), which control cellular glucose uptake, are expressed in the endometrial stroma [19]. *SLC2A1* (*GLUT1*) mRNA was found to be the most abundant in stromal cells and its expression was shown to be required for *in vitro* differentiation of human stromal cells [19]. Interestingly, women with idiopathic infertility were shown to have significantly lower levels of *SLC2A1* in their stroma than controls with non-endometrial infertility [20], suggesting that decreased glucose uptake might contribute to impaired stromal differentiation and lead to impaired endometrial function and infertility in these patients. The expression of *SLC2A8* (*GLUT8*) mRNA in both mouse and human stromal cells was reported to increase dramatically in response to the hormonal changes that occur during the process of implantation. The ovaries of *Slc2a8* (-/-) mice exhibited abnormal metabolism and ATP production. Down regulation of *SLC2A8* expression in human endometrial stromal cells resulted in impaired differentiation [21], suggesting that the GLUTs play an important role in meeting the increased demand for glucose during this process. Our gene expression profiling studies revealed

that the expressions of glucose transporters *SLC2A3* (*GLUT3*) and *SLC2A11* (*GLUT10*, *GLUT11*) were modestly up regulated by PRB, suggesting that P signaling might be involved in the regulation of glucose uptake. Further studies, addressing the functional roles of these GLUTs, are necessary to clarify the link between PR signaling and regulation of glucose uptake.

In addition to the GLUTs, our microarray analysis indicated that the insulin receptor substrate 2 (*IRS2*), an adaptor substrate for insulin and insulin-like growth factor-1 receptors, is a P-regulated gene. Insulin signaling is a major regulator of cellular glucose uptake. Binding of insulin to its receptor tyrosine kinase triggers phosphorylation of insulin receptor substrates (*IRS* family). The phosphorylated tyrosine residues on the *IRS* proteins become docking sites for several downstream signaling molecules, such as *PI3K* and *GRB3*. In muscle cells and adipocytes, signaling via these pathways control critical processes, including cell survival, glycogen synthesis/breakdown and translocation of *GLUT4* to plasma membrane. Certain reproductive disorders are associated with impaired insulin metabolism. For example, polycystic ovary syndrome (*PCOS*), a female endocrine disorder, is characterized by cystic ovaries, excessive amounts of androgenic hormones, and decreased or no ovulation. *PCOS* patients also have higher rates of insulin resistance, miscarriage and obesity [22, 23]. Although the major cause of the infertility is thought to be an ovarian defect, the reasons for the high incidence of miscarriage in *PCOS* remain unclear. Defects in certain components of the insulin-signaling pathway, including excess phosphorylation of the insulin receptor and lower levels of *IRS1* and *GLUT4*, have been reported in the endometrium of *PCOS* patients with hyperinsulinemia [24, 25], suggesting that impaired insulin signaling may lead to impaired endometrial function and fertility problems.

Our results indicated that *IRS2* serves as an important regulator of energy metabolism during decidualization. Using ChIP-seq and gene expression analyses, we identified *IRS2* as a potential direct target of both *PRA* and *PRB*. These findings are consistent with previous reports indicating a role of P in regulating *IRS2* expression, which was further linked to insulin-like growth factor signaling [26-28]. In human endometrial stromal cells, expression and phosphorylation of *IRS2* increased during *in vitro* decidualization [29], suggesting that it might play a critical role during the differentiation process.

Development of the *Irs2* null mouse underlined the importance of energy metabolism in regulation of female fertility [30]. *Irs2* (-/-) female mice were infertile. Infertility was thought to be due to an ovarian defect. Levels of E, P, and the gonadotropins were also low in these animals. The status of decidualization in the *Irs2* (-/-) mice is not known. In human endometrial stromal cells, we showed that the loss of *IRS2* expression led to impaired differentiation, suggesting an important role for *IRS2* in human decidualization. We propose that *IRS2* is a critical link between PR signaling and energy metabolism during stromal differentiation. *IRS2* may be involved in localization of glucose transporters to the cell membrane to enable the endometrial stromal cells to take up enough glucose to meet the increased energy needs during their proliferation and differentiation. Future studies, addressing the level and localization of glucose transporters in stromal cells lacking *IRS2*, would be useful to test this possibility. *IRS2* may also be involved in the regulation of genes controlling glucose metabolism. In a recent study, it was shown that *IRS2* is required for the expression of glucokinase and glucokinase regulatory protein in the liver [31]. We will, therefore, investigate whether *IRS2* regulates the expression of key enzymes involved in glucose metabolism, such as hexokinase, phosphofructokinase and pyruvate kinase, during human stromal decidualization.

REFERENCES

1. Stender, J.D., et al., *Estrogen-regulated gene networks in human breast cancer cells: involvement of E2F1 in the regulation of cell proliferation*. Mol Endocrinol, 2007. **21**(9): p. 2112-23.
2. Wu, Y. and S.W. Guo, *Inhibition of proliferation of endometrial stromal cells by trichostatin A, RU486, CDB-2914, N-acetylcysteine, and ICI 182780*. Gynecol Obstet Invest, 2006. **62**(4): p. 193-205.
3. Lydon, J.P., et al., *Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities*. Genes Dev, 1995. **9**(18): p. 2266-78.
4. Franco, H.L., et al., *In vivo analysis of progesterone receptor action in the uterus during embryo implantation*. Semin Cell Dev Biol, 2008. **19**(2): p. 178-86.
5. Satokata, I., G. Benson, and R. Maas, *Sexually dimorphic sterility phenotypes in Hoxa10-deficient mice*. Nature, 1995. **374**(6521): p. 460-3.
6. Mulac-Jericevic, B. and O.M. Conneely, *Reproductive tissue selective actions of progesterone receptors*. Reproduction, 2004. **128**(2): p. 139-46.
7. Attia, G.R., et al., *Progesterone receptor isoform A but not B is expressed in endometriosis*. J Clin Endocrinol Metab, 2000. **85**(8): p. 2897-902.
8. Wu, Y., et al., *Promoter hypermethylation of progesterone receptor isoform B (PR-B) in endometriosis*. Epigenetics, 2006. **1**(2): p. 106-11.
9. Kumar, N.S., et al., *Selective down-regulation of progesterone receptor isoform B in poorly differentiated human endometrial cancer cells: implications for unopposed estrogen action*. Cancer Res, 1998. **58**(9): p. 1860-5.
10. Clarke, C.L. and R.L. Sutherland, *Progestin regulation of cellular proliferation*. Endocr Rev, 1990. **11**(2): p. 266-301.

11. Li, Q., et al., *The antiproliferative action of progesterone in uterine epithelium is mediated by Hand2*. Science, 2011. **331**(6019): p. 912-6.
12. Lee, K.Y., et al., *Bmp2 is critical for the murine uterine decidual response*. Mol Cell Biol, 2007. **27**(15): p. 5468-78.
13. Li, Q., et al., *WNT4 acts downstream of BMP2 and functions via beta-catenin signaling pathway to regulate human endometrial stromal cell differentiation*. Endocrinology, 2013. **154**(1): p. 446-57.
14. Hsieh-Li, H.M., et al., *Hoxa 11 structure, extensive antisense transcription, and function in male and female fertility*. Development, 1995. **121**(5): p. 1373-85.
15. Lim, H., et al., *Hoxa-10 regulates uterine stromal cell responsiveness to progesterone during implantation and decidualization in the mouse*. Mol Endocrinol, 1999. **13**(6): p. 1005-17.
16. Taylor, H.S., et al., *Sex steroids mediate HOXA11 expression in the human peri-implantation endometrium*. J Clin Endocrinol Metab, 1999. **84**(3): p. 1129-35.
17. Taylor, H.S., et al., *HOXA10 is expressed in response to sex steroids at the time of implantation in the human endometrium*. J Clin Invest, 1998. **101**(7): p. 1379-84.
18. Frolova, A.I. and K.H. Moley, *Glucose transporters in the uterus: an analysis of tissue distribution and proposed physiological roles*. Reproduction, 2011. **142**(2): p. 211-20.
19. Frolova, A.I. and K.H. Moley, *Quantitative analysis of glucose transporter mRNAs in endometrial stromal cells reveals critical role of GLUT1 in uterine receptivity*. Endocrinology, 2011. **152**(5): p. 2123-8.
20. von Wolff, M., et al., *Glucose transporter proteins (GLUT) in human endometrium: expression, regulation, and function throughout the menstrual cycle and in early pregnancy*. J Clin Endocrinol Metab, 2003. **88**(8): p. 3885-92.

21. Adastra, K.L., et al., *Slc2a8 deficiency in mice results in reproductive and growth impairments*. Biol Reprod, 2012. **87**(2): p. 49.
22. Carmina, E. and R.A. Lobo, *Polycystic ovary syndrome (PCOS): arguably the most common endocrinopathy is associated with significant morbidity in women*. J Clin Endocrinol Metab, 1999. **84**(6): p. 1897-9.
23. Sivayoganathan, D., et al., *Full investigation of patients with polycystic ovary syndrome (PCOS) presenting to four different clinical specialties reveals significant differences and undiagnosed morbidity*. Hum Fertil (Camb), 2011. **14**(4): p. 261-5.
24. Fornes, R., et al., *Changes in the expression of insulin signaling pathway molecules in endometria from polycystic ovary syndrome women with or without hyperinsulinemia*. Mol Med, 2010. **16**(3-4): p. 129-36.
25. Dunaif, A., *Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis*. Endocr Rev, 1997. **18**(6): p. 774-800.
26. Vassen, L., W. Wegrzyn, and L. Klein-Hitpass, *Human insulin receptor substrate-2 (IRS-2) is a primary progesterone response gene*. Mol Endocrinol, 1999. **13**(3): p. 485-94.
27. Cui, X., et al., *Progesterone crosstalks with insulin-like growth factor signaling in breast cancer cells via induction of insulin receptor substrate-2*. Oncogene, 2003. **22**(44): p. 6937-41.
28. Yudt, M.R., et al., *Selective and opposing actions of progesterone receptor isoforms in human endometrial stromal cells*. Mol Cell Endocrinol, 2006. **247**(1-2): p. 116-26.
29. Ganeff, C., et al., *The IGF system in in-vitro human decidualization*. Mol Hum Reprod, 2009. **15**(1): p. 27-38.
30. Burks, D.J., et al., *IRS-2 pathways integrate female reproduction and energy homeostasis*. Nature, 2000. **407**(6802): p. 377-82.

31. Roncero, I., et al., *Insulin-receptor substrate-2 (irs-2) is required for maintaining glucokinase and glucokinase regulatory protein expression in mouse liver*. PLoS One, 2013. **8**(4): p. e58797.