

INTERACTION OF TRANSCRIPTION FACTORS IN REGULATION OF HUMAN
CHORIONIC GONADOTROPIN (HCG) ALPHA SUBUNIT GENE PROMOTER
ACTIVITY

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Academic Abstract

Chorionic gonadotropin (CG), which in higher primates, including the humans, is considered the major embryonic signal for maternal recognition of pregnancy, is a glycoprotein hormone synthesized and secreted by the trophoblast. The upsurge in secretion of CG during implantation and its binding to the luteinizing hormone (LH) receptor extends the life-span of the corpus luteum, thereby allowing a steady supply of ovarian progesterone required to maintain pregnancy. CG is a heterodimer, consisting of an alpha (α) - and a beta (β) -subunit. The α -subunit is common to all the glycoprotein hormones, while the β subunit is unique and accounts for biological specificity. The transcriptional control mechanisms responsible for CG subunit expression in human trophoblast cells have been extensively studied and several key regulatory elements identified for both subunit genes. Here I focused on *CGA* and on three transcription factors, ETS2 and DLX3, which transactivate the gene, and the POU-domain factor, OCT4, which silences it. All experiments were performed in human choriocarcinoma cells (JAR). In particular, I investigated the mechanism underlying OCT4-mediated repression of *CGA*, concentrating on the interaction between OCT4 and ETS2. ETS2 binds to two overlapping sequences in the proximal part of the *CGA* promoter and modestly up-regulates its activity. This transactivation is reversed in a dose-dependent manner by co-expression of OCT4. Mutation of the OCT4 binding site did not alter its silencing activity. An ETS2-DNA complex was formed in absence of OCT4 but not in its presence, suggesting that OCT4 silences *CGA* promoter by a squelching mechanism. Although OCT4-mediated repression of ETS2 transactivation occurs independently of its

ability to bind to a binding sequence in the proximal CGA promoter, mutation of this site lowered basal promoter activity. Here I demonstrate that the homeobox transcription factor, DLX3 occupies an overlapping portion of this site. Although DLX3 alone has only weak transactivation ability, it becomes a powerful transactivator in the presence of ETS2. The two factors interact physically, but this interaction appears to be interrupted by the co-expression of OCT4. The observations may explain why CGA production only begins to increase as OCT4 expression is down-regulated in the trophoblast and extends the notion that pluripotency of the inner cell mass and epiblast of the developing embryo is mediated in part by the ability of OCT4 to squelch transcription factors driving lineage determination.

To examining this role of OCT4 further, I determined whether its stable expression in JAr cells could partially reprogram the cells to a less differentiated state. I made use of two OCT4-expressing JAr cell lines. These cells produce much reduced quantities of both CGA and CGB. A microarray analysis, which compared the two OCT4-positive lines with two controls, showed that while a number of genes were down-regulated, including *CGA* and *CGB*, a majority of effected genes were up-regulated. The latter included several genes normally considered to be associated with a pluripotent phenotype, including *DPPA2*, *3 & 4* and *ZFP42 (REX1)*. Unexpectedly, the gene for *CDX2*, a transcription factor normally considered to be down-regulated by OCT4, showed increased mRNA expression. Together, the data suggested that the forced, though relatively low, expression of OCT4 in JAr cells was capable of converting them to a less differentiated state, possibly closer to their trophoblast stem cell origin.

Chapter I

Literature review

Early embryonic development:

In humans, fertilization occurs in the fallopian tube within 24 to 48 h of ovulation. During the passage through the fallopian tube, the embryo undergoes initial stages of development, beginning from a fertilized ovum or zygote to a ball of 12 to 16 cells, also known as a morula. Upon entering the uterine cavity, the morula undergoes a transition towards a fluid – filled inner cavity within the mass of cells (Fig. 1). This transition of the morula leads to the formation of the blastocyst that is comprised of a layer of cells on the surface, which later becomes the trophoblast (the major fetal component of the placenta), and the inner cell mass (ICM), which becomes the embryo proper (Rossant and Vijn 1980; Johnson and Ziomek 1981). The transformation of the morula to a blastocyst represents the first cell division event in early mammalian development. Within a span of 3 days, the embryo hatches from the zona pellucida (a non – adhesive protective glycoprotein coating) to expose its outer trophectoderm layer, an epithelium that is partially syncytial (Norwitz et al. 2001; Red-Horse et al. 2004). In mouse and human, the ICM is internal and is covered by polar trophectoderm on one side and blastocoel fluid covering the other side. In swine and ruminants, at the blastocyst stage, the embryo looks very similar and structurally organized like the blastocyst of human and mouse. It is only later that the embryonic disc becomes exposed as described below. Once the blastocyst hatches from the zona, the overlying trophoblast begins to degenerate and exposes the embryonic disc to extraembryonic environment (Flechon et al. 2004). In human and

mouse, hatching is immediately followed by implantation, a complex process that involves invasion and the formation of the early placenta and is accompanied by the establishment of a complex dialogue between embryonic and maternal cells that allows the mother to accommodate the growing conceptus. In the human, implantation is a crucial process that sequesters the embryo within the uterine wall. However, there is considerable variation across species with regard to extent of invasion, the morphology of the placenta, and the nature of the biochemical cross – talk that occurs (Norwitz et al. 2001; Paria et al. 2002).

Implantation in humans involves three stages. The initial event of attachment of the blastocyst to the uterine epithelium is called apposition. The second stage of stable adhesion is characterized by increased physical interaction between the blastocyst and the uterine epithelium. In the final stage, the invasion begins, and syncytial trophoblast cells begin to penetrate the uterine epithelium and invade the underlying stroma. A successful implantation leading to the establishment of pregnancy requires a receptive uterus that is hormonally “in phase” with the conceptus (Paria et al. 2001). The receptive state is defined by the limited time when the uterine environment is conducive to blastocyst acceptance and implantation. This limited window of receptivity is followed by a spontaneous progression towards a non – receptive state when the uterine environment becomes hostile to the blastocyst survival. In rodents, the receptivity lasts for 24 to 36 h as opposed to longer window in primates (Red-Horse et al. 2004). Following successful attachment and invasion, the human trophoblast begins to occupy the underlying maternal capillary space resulting in formation of a hemochorial placenta (Fazleabas and Strakova 2002). Endometrial modifications occur that create a uterine environment that is

favorable to the development of the embryo as well as immunologically tolerant of the semi-allograft (the embryo) (Loke et al. 1995). Synchronization between the development of the blastocyst and modification in the endometrium is crucial for successful pregnancy. Among normal fertile couples, the pregnancy loss rate after blastocyst formation has been estimated to be 15 – 19 % (Ezra and Schenker 1995). Implantation failures after in vitro fertilization (IVF) are also high.

Delayed implantation is a process by which implantation is postponed for a certain period of time. It occurs naturally in a variety of species where the uterus remains in a quiescent state and the embryos at the blastocyst stage become dormant (Mead, 1993). Recent evidence suggests that a short delay in attachment reaction produces an adverse ripple effect throughout pregnancy, with defective placentation and retarded development of fetuses, ultimately giving rise to a poor pregnancy outcome (Song et al. 2002). This complex process of implantation involves spatiotemporally regulated endocrine, paracrine and autocrine modulators that span cell-cell and cell-matrix interactions. The embryo also functions as an active unit with its own molecular program of cell growth and differentiation (Wilcox et al. 1988). Thus deficiencies in uterine receptivity, poor embryo development and an inadequate embryo-uterine dialogue can compromise conceptus survival and hence fertility.

Placentation:

In ancient times, the placenta was considered to resemble a funnel (flattened) cake. The placenta begins to develop upon implantation of the blastocyst into the maternal endometrium. The placenta is generally considered to comprise of both fetal and maternal

tissues, since the two interact physically. However, placentae vary greatly in gross morphology across mammals and the extent to which the fetal component “invades” the maternal uterine endometrium. The placenta forms an interface between mother and fetus, providing support and facilitating the exchange of various nutrients and gases. The placenta is also considered as an endocrine organ, secreting steroids and necessary hormones that play a major role in allowing maternal physiology to adapt to the pregnancy and ensuring maternal sustenance for the growing fetus. In the majority of pregnancies, the embryo/fetus is genetically different from the mother because of carrying a complement of paternal alleles, although exceptions do occur, e.g. in the case of inbred strains of mice. This situation comprises one of the major paradoxes of contemporary immunology, raising the question of how the genetically different embryo/fetus and mother co-exist during gestation (Telugu et al, 2007).

Classification of placental types:

Placental types can be classified based on:

- a) Gross morphology that includes shape and distribution of the chorionic tissues that interact with the uterine tissues.
- b) The number of cell-types that separate the maternal and fetal tissues.

Based on morphology, placentas can be classified into four general types:

1. *Diffuse placentae:*

Diffuse placental types are found in pigs and horses. In pigs and related species, the placenta is completely non-invasive. The chorionic villi are closely packed, convoluted and distributed all over the uterine epithelium. The villi facilitate the exchange of nutrients between maternal and fetal circulation. The equine placenta possesses localized regions of intimate contact called microcotyledons, where much of nutrient and gas exchange occurs, but as pregnancy progresses some trophoblast cells detach and invade the endometrium to form pockets of binucleated cells called endometrial cups which produce hormones including pregnant mare gonadotrophin, a form of luteinizing hormone (Fig.2) (Telugu et al, 2007).

2. *Cotyledonary placenta:*

Cotyledonary placenta is a feature of ruminant ungulates. The cotyledons are comprised of vascular villous trophoblast. These villi intercalate into the caruncles of uterine endometrium. Together, the combination of caruncles and cotyledons are called the placentome, where maternal-fetal exchanges take place (Fig. 2) (Telugu et al, 2007).

3. *Zonary placenta:*

All carnivores possess zonary placenta where a band of chorion surrounding the middle of the fetus forms a zone of tight contact with the maternal uterus. The chorionic villi aggregate to form a broad band that forms circles around the center of the chorion. Such zones may be complete circles (such as those in dogs and cats) or incomplete (such as those in bears and seals)(Telugu et al, 2007). Zonary placenta is thought to be formed

from diffuse placenta in which the villi at the ends regress, leaving only those in the center to function. At the edges of the zonary placenta is the hemophagous organ, which is green. The color is due to the degradation of hemoglobin into biliviridin. This provides iron for the developing fetus. (Gilbert, 2002) (Fig. 2).

4. *Discoid placentae:*

A discoid placenta consists of one or more distinct discs that comprise regions of fetal chorion that interface with uterine tissues. Higher primates, including humans, and rodents have discoid placental types (Fig. 2) (Telugu et al, 2007).

As stated above, placentae have also been classified according to the number of cell layers between the fetal and maternal blood supplies. The maximum total of cell layers is six. There are three layers of fetal extraembryonic membranes in the chorioallantoic placenta of all mammals, all of which are components of the mature placenta. These fetal components consist of the endothelium lining of allantoic capillaries, connective tissue in the form of chorioallantoic mesoderm and the outermost layer of fetal membranes derived from trophoblast, the chorionic epithelium. There are also a potential maximum of three layers on the maternal side: the endothelium lining endometrial blood vessels, connective tissue of the endometrium and the endometrial epithelial cells. The number of maternal layers that are retained in the process of placentation varies among species.

Four major types of classifications have been made on the basis of cell layers:

1. *Hemochorial placenta:*

The hemochorial placenta is invasive and occurs when chorionic trophoblast penetrates the maternal uterine epithelium, the underlying stroma and the endothelium of maternal vasculature to establish direct contact with the maternal blood. The three cell-types between the maternal and fetal blood are fetal trophoblast, fetal connective tissue and fetal endothelial cells. Hemochorial placentae are found in rodents, rabbits and higher primates, including humans (Fig.3) (Telugu et al, 2007).

In humans, the blastocyst enters the uterus from the fallopian tube by day 4 – 5 at about the time the blastocyst forms and attaches trophoctoderm on the side of the embryonic pole to either the dorsal or ventral uterine wall (Norwitz et al. 2001; Paria et al. 2002). During implantation, the cytotrophoblast cells surrounding the embryo undergo rapid proliferation and differentiate to form a multinucleated syncytiotrophoblast. These multinucleated cells invade the luminal epithelium as well as the underlying stroma to become completely embedded within the endometrial stroma (Paria et al. 2002). Gradually, intercellular spaces, also known as lacunae begin to appear within the syncytiotrophoblast cell layers (Enders and Welsh 1993). These intercellular spaces eventually become filled with maternal blood due to the rupture of maternal endothelial cells (Enders and King 1991). The human conceptus gradually progresses through various developmental stages, which includes formation of yolk sac, amnion as well as allantoic mesoderm. The amnion is one of the four extraembryonic fetal membranes. It eventually fuses with the chorion (the outermost tissue covering the fetal membrane) to form the amniochorion, a distinguishing characteristics in human placentation (Sadler, 2000). Another distinctive feature of human placentation is the early morphogenesis of

the chorion into chorioallantois (Mossman, 1987). During the early stages of development, a subpopulation of the cytotrophoblast cells leave the chorionic plate, penetrate the syncytiotrophoblast cell layer and gives rise to anchoring villi that make contact with the functional maternal deciduas (Castellucci, 1990) (Fig.4). Extravillous trophoblast cells also invade maternal spiral arteries and bring them under the control of the conceptus, allowing greater quantities of blood to flow through the arteries to the sites of nutrient and gas exchange.

2. *Endotheliochorial placenta:*

This type of placenta is similar to hemochorial placenta in terms of invasiveness. However, the trophoblast does not come into direct contact with the maternal blood. On the contrary, the trophoblast associates with the outer surface of endothelium of the maternal capillary network, but does not penetrate the vessels themselves. There are four cell-layers separating maternal and fetal circulations. This type of placenta is typically found in carnivores and insectivores (Fig. 3) (Telugu et al, 2007).

3. *Epitheliochorial placenta:*

The epitheliochorial placenta is the least invasive type. The luminal epithelium of the endometrium as well as the epithelium of the chorionic villi remains intact. There are six cell-layers separating the maternal and fetal circulations. This type of placenta is found in pigs, horses, whales, and some primates (Fig.3) (Telugu et al, 2007).

4. *Synepitheliochorial placenta:*

This type of placenta is similar to the epitheliochorial placenta type, except there is a limited amount of invasion. Specialized binucleate trophoblast cells fuse with uterine epithelial cells. In some species, such as sheep and goat, continued migration and fusion lead to the formation of an extensive fetal-maternal syncytial layer in early pregnancy, but no further penetration of trophoblast occurs. In cattle, the fusion of binucleate trophoblast cells with uterine epithelial cells results in formation of short-lived trinucleate cells. Usually, five to six cell-layers separate the fetal and maternal circulatory system. This type of placenta is typically found in ruminants (Fig. 3) (Telugu et al, 2007).

Placental hormones and polypeptides:

As mentioned earlier, placental morphology varies considerably across species. Often a range of placental types are found within particular orders of mammals. These different types of placentae produce a wide variety of hormones and other factors that play a significant role in establishment and maintenance of successful pregnancy, most probably in part because the structural barriers to biochemical communication also vary. Some of these hormones are considered as primary signals for maternal recognition of pregnancy since they inform the mother that she is pregnant and prepare her and her uterus to accept and nourish the growing conceptus. Some of these factors aid in nutrient exchange between mother and the fetus and can also maintain a successful pregnancy by preventing the regression of corpus luteum (CL), thereby maintaining a steady source of progesterone secretion. Some of these important hormones and proteins that play a significant role throughout the pregnancy in various species are as follows:

Human Chorionic Gonadotropin (hCG):

Higher primates, including humans, secrete a chorionic gonadotropin (CG), which plays a significant role in maternal recognition of pregnancy, maintenance of progesterone secretion from CL (by preventing the latter from regressing) and other effects on the mother, such as immunomodulation, which are incompletely understood (Cameo et al. 2004; Licht et al. 2007). CG creates a favorable uterine environment for the conceptus during implantation (blastocyst attachment to the endometrium). It facilitates the implantation process by inducing vasodilation of myometrial blood vessels and relaxation of the smooth muscles (Cameo et al. 2004; Licht et al. 2007). In humans, CG can be detected in the maternal serum after 7-8 days after gestation. Detection of CG in urine takes a few more days and is the basis of the common test for pregnancy. In the maternal serum, the level of CG peaks at 7-9 weeks of gestation, followed by a gradual decline (Pittaway et al. 1985; Alfthan and Stenman 1996; Muyan et al. 1996; Licht et al. 2007).

Interferon – τ (IFN- τ):

IFN- τ is considered as primary signal for maternal recognition of pregnancy in ruminants (Roberts 1993). Production of IFN- τ begins between day 10-25 of gestation and peaks around day 14-16 in bovine (Roberts et al. 1999; Spencer and Bazer 2004). IFN- τ prolongs the lifespan of CL by inhibiting the release and possibly also the production of the luteolytic factor Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). It is known to down-regulate the oxytocin receptor, possibly via reducing expression of uterine estrogen receptors (Roberts et al. 1999; Spencer and Bazer 2004), but may also regulate the

expression of genes encoding various enzymes involved in prostaglandin synthesis and metabolism (Chen et al. 1992).

Pregnancy – associated glycoproteins (PAGs):

PAGs are classified as inactive members of the aspartic proteinase family. These proteins are related to pepsin, chymosin and cathepsin (Xie et al, 1991). Various isoforms of PAGs are produced from the ruminant placenta. PAGs have the potential to be used for clinical studies in pregnancy determination in cattle (Green et al, 2005; Sousa et al, 2006). These proteins can be detected in pregnant maternal serum or plasma (Perenyi et al. 2002; Karen et al. 2003; Gonzalez et al. 2004).

Placental lactogen and Prolactins:

Various factors related to the pituitary prolactin and growth hormone are produced from ruminant (Dietz et al, 1992), rodent (Soares and Talamantes, 1985) and human placenta (Handwerger and Freemark, 2000), and most probably from other species as well (Byatt et al. 1992; Forsyth, 1994; Anthony et al. 1995). These compounds are classified as placental lactogens (PL) or prolactin-related compounds. These proteins may play a major role in regulating maternal and fetal metabolism and in regulating the immune response. For example, placental lactogen in cattle may increase fetal growth by increasing the secretion of insulin – like growth factor (IGF) in the conceptus (Byatt et al. 1992) and uterine milk proteins (UTMs) in rodents may help control the activity of natural killer cells in the endometrium (Liu and Hansen, 1993).

Equine chorionic gonadotropin (Pregnant Mare Serum Gonadotropin):

At around day 35-37 of pregnancy, eCG or PMSG is secreted from the equine endometrium (Martinuk et al. 1991; Hoppen, 1994; Allen, 2001). In reality eCG is a form of luteinizing hormone (LH) but is glycosylated differently than LH produced in the pituitary. Unlike other CGs found in higher primates, including humans, eCG is not considered as the primary signal for maternal recognition of pregnancy. This hormone is secreted by the invading binucleate cells that form the chorionic cups embedded in the endometrium after placentation and stimulates additional follicular developments (Hoppen, 1994). The secretion of eCG peaks during 60 to 80 days after gestation and gradually declines by day 130 of gestation as the cups regress. The role of eCG appears to be that of stimulating the formation and maintenance of extra CL and hence progesterone synthesis during the first trimester of an equine pregnancy (Martinuk et al, 1991).

Steroid hormones:

Continued synthesis of progesterone by the CL is crucial for maintenance of a successful pregnancy, since it is required to target progesterone receptors in the endometrium and keep the tissue in a state that supports the development and growth of the conceptus (Hoffmann and Schuler 2002; Ousey et al. 2003). Interruption of progesterone production terminates mammalian pregnancies. Although the CL is the primary structure for production of progesterone in early pregnancy, the placenta often begins to play a major role in other species, e.g. sheep, cattle and horses, as pregnancy progresses (Silver, 1994; Nicklin et al, 2007).

Like progesterone, estrogen is equally important in maintaining successful pregnancy. Estrogen is produced by the placenta of various domestic animals (Choi et al. 1997). In pigs, estrogen is considered as the primary signal for maternal recognition of pregnancy (Choi et al. 1997). It prevents luteolysis by redirecting endometrial $\text{PGF}_{2\alpha}$ into the uterine lumen rather than promoting its release into the maternal blood supply.

Trophoblast differentiation:

Trophectoderm is the precursor of placental trophoblast and is required for early embryonic development. It plays an important role in transporting solute and fluids (nutrients), attachment of conceptus to the uterine wall (implantation) and providing a protective layer around the inner cell mass. Early trophoctoderm is invasive in some species where implantation occurs just after hatching of the zona, e.g. mouse and human. It is also a source of various pregnancy-associated hormones, such as IFN - Tau in ruminants and chorionic gonadotropin (CG) in higher primates. Trophoblast thus plays a significant role in triggering the maternal responses towards the presence of the conceptus in the uterus (Roberts et al. 1996). Trophectoderm is the first overtly differentiated tissue of the embryo and is visible in blastocysts as a polarized epithelium enclosing a fluid filled cavity (blastocoel) and the inner cell mass. This single layer of outer layer subsequently gives rise to the trophoblast lineage that in turn forms most of the fetal component of the mature placenta (Roberts et al, 1996). The trophoblast forms a functional bridge between the mother and the fetus and thereby performs a majority of absorptive, immunoprotective and endocrinological functions of the placenta (Roberts et al. 2004).

A combinatorial expression of various transcription factors regulates the patterning of all cell lineages, including the emerging trophoderm and its more mature sub-lineages. The expression of some of these transcription factors is probably part of a pre-configured program while the expression of others may be controlled by the presence of small molecules, such as nutrients or oxygen, or growth factors, thereby establishing the boundaries for cell lineage specification within the early embryo (Roberts et al. 2004). In humans, the trophoderm undergoes differentiation to give rise to mononuclear cytotrophoblast and a series of more mature lineages such as multinucleate syncytiotrophoblast, columnar cells and other forms of extra-villous cytotrophoblast, all of which appear to require the expression of an orderly program of transcription factor networks (Cross et al, 2002). Although both the human and murine placentae are hemochorial and have many common features, they are morphologically quite distinct (Rossant and Cross 2001). In the mouse, the outer layer is comprised of trophoblast giant cells that play a significant role in implantation and invasion into the uterus. These cells share a lot of similarity with the extravillous cytotrophoblast cells in humans (Georgiades et al. 2002; Cox et al, 2009).

In ruminants, like other placental mammals, the formation of placental trophoblast involves differentiation lineages derived from trophoderm cells. However in ruminants and ungulates in general, extensive trophoblast development occurs well before the placenta develops (Degrelle et al. 2005). The conceptus elongates, largely as the result of extensive growth of the trophoblast and underlying extra-embryonic endoderm, before definitive attachment of the trophoderm to the uterine epithelium and expansion of the allantoic sac is initiated (Degrelle et al, 2005). It remains unclear whether the

mononucleated cells of trophoblast consist of multiple differentiated cell types with specialized function or whether they are all part of a relatively uniform tissue layer. Thus, there are fundamental differences between development of trophoblast in the human, mouse and ruminants. In the mouse, in particular, the segregation of the embryonic as well as the extraembryonic lineages is initiated at the time of implantation. Molecular programming of trophoblast differentiation may also differ between mouse and bovine embryos (Degrelle et al. 2005).

Models and cell-lines to study trophoblast research:

The trophoblast cells of the placenta play a significant role in setting up a communication between fetus and the mother. Thus, it is important to understand the molecular mechanisms underlying placental development. Trophoblast research is performed on in vitro models and has generally involved primary cell cultures derived from differentiated trophoblast, tumor cell lines, rather than in-vivo studies of animal models. The mouse has been used extensively as a model to study human placentation, in large part because of its convenience as a laboratory animal and more recently because genetic knock out studies have revealed the presence of genes required for proper placental development. (Carter et al, 2007). Other models such as guinea pigs and primates have also been used to study placental development, although genetic models are not available in such species (Carter et al, 2007). A combination of various models is required to identify the conserved mechanisms as well as the subcellular events that are critical for human placentation.

Trophoblast cell lines are generated from normal trophoblast or from choriocarcinoma cells. Choriocarcinoma cells are malignant tumors arising within

placental trophoblast, which, as discussed earlier, is comprised of a mixed population of cells. Choriocarcinoma cells are believed to arise from dividing cellular intermediates resembling the cytotrophoblasts. These cell lines have been used extensively as a tool to understand trophoblast cell biology and endocrine function, as well as placental development (King et al. 2000). The most commonly used choriocarcinoma cells for trophoblast studies are JAr, JEG and BeWo cells. These cell lines are morphologically similar, but not identical, and have served as a convenient in vitro model for studying various cellular activities as well as regulation of trophoblast – specific genes. Choriocarcinoma cell lines are not uniform and tend to differentiate in culture and consist of a mixed population of cells that includes cytotrophoblast, a few syncytiotrophoblasts, and intermediate cell types (Taylor et al. 1991). JAr cells, in particular, are dominated by mononucleated small cells with features of early cytotrophoblast. They synthesize hCG in only small amounts (White et al. 1988). The BeWo cell lines are believed to comprise extravillous cytotrophoblasts. JEG cells probably were originally derived from BeWo cells and also express CG and placental lactogen (Kohler and Bridson 1971). Although these cell lines, being of human origin, do not express many of the genes characteristic of trophoblast from other species such as interferon tau (IFNT), trophoblast Kunitz domain protein – 1 (TKDP-1) and pregnancy associated glycoproteins (PAG), these cells appear to have the necessary transcription factors that are permissive for the ectopic expression of the genes after they are transfected with appropriate vectors. (MacLean et al, 2003; Chakrabarty et al, 2006; Ezashi et al. 1998; Ezashi et al. 1998; Szafranska et al. 2001).

Maternal recognition of pregnancy and human chorionic gonadotropin (hCG):

Establishment of successful pregnancy involves three important early phases: fertilization, maternal recognition of pregnancy and successful implantation. One important aspect of maternal recognition of pregnancy is the physiological process whereby the conceptus (embryo and its membrane) signals its presence to the maternal system and thereby increases the lifespan of the corpus luteum (CL) by preventing its regression (Roberts, Xie et al. 1996). The CL plays a significant role in regulation of the estrus cycle and maintenance of pregnancy. It is also considered as the final form of a developing follicle and is the major endocrine component of the ovary. A necessary part of the maternal response to signals from the conceptus is the continued production of progesterone, secreted by the corpus luteum. CL also produces a variety of other hormones such as estradiol, relaxin, inhibin A and B (Tsigkou et al, 2008), certain cytokines and prostaglandins (Sunder and Lenton, 2000). CL is important for preparation of the endometrium for implantation. Its removal in early pregnancy of all known placental mammals results in miscarriage. In higher primates, including humans, CG is the glycoprotein hormone that plays a dominant role in maintaining a constant supply of progesterone, which is accomplished by extending the lifespan of the corpus luteum (CL) and preventing its normal cyclic regression (Duncan 2000). In addition to its luteotrophic function, CG also prevents the luteolysis by maintaining luteal blood flow, preventing tissue remodeling by controlling the activity of matrix metalloproteinases (MMPs) and the influx of macrophages (Duncan 2000), and may prevent apoptosis by increasing the Bcl2/Bax ratio (Sugino, Suzuki et al. 2000). Due to the limited life span of the CL, all these functions are maintained until the luteo-placental shift occurs in humans, i.e. until

the time that the placenta produces sufficient progesterone to maintain the pregnancy (Schindler, 2005).

CG is a heterodimer, consisting of an alpha (α) and a beta (β) subunit. The α -subunit is common to all glycoprotein hormone family members (TSH, LH, FSH). However, the β -subunit is unique and is responsible for biological specificity of the hormone (Fig.6). All the glycoprotein hormones are predicted to share similarity in their tertiary structures (Cameo, Srisuparp et al. 2004), yet, with the exception of LH and CG, bind to different receptors. CG is synthesized and secreted primarily, but not exclusively, from the multi-nucleated non-dividing syncytiotrophoblast cells. The syncytiotrophoblast cells also secrete various other placental hormones such as placental lactogen, chorionic somatomammotropin and steroids (Lopata 1996). Traces of *CGB* mRNA can be detected in an early 6-8-cell stage of the human embryo (Alfthan et al, 1996). However, measurable CG hormone produced by cultured embryos cannot be detected in the culture medium by either bioassays or immunoassays until around day 7 (Alfthan and Stenman 1996). CG can be measured in maternal serum and urine ~ 8 – 10 days after conception. Its concentration increases exponentially with a doubling time of 1.5 days during 4th week and 2.5 days around 5th week after conception. The concentration of CG reaches its peak around week 6 after ovulation, subsequently declines and reaches a lower plateau that persists throughout pregnancy (Fig.5) (Pittaway, Reish et al. 1985; Alfthan and Stenman 1996; Muyan, Furuhashi et al. 1996; Licht, Fluhr et al. 2007). The free CGB subunit is detected in the urine sample and its concentration during early pregnancy increases by 5 fold at term (Cole et al, 1984; Ozturk et al, 1987). Free CGB subunit accounts for only ~ 3 % of the total hCG detectable by immunoassays in maternal

circulation throughout pregnancy (Cole, Kroll et al. 1984; Ozturk, Bellet et al. 1987). In plasma, the concentration of free CGB is higher than free CGA in 2 – 5 weeks of pregnancy, but becomes lower than CGA by the third trimester (Cole, Kroll et al. 1984).

Structural analysis of CGA and CGB subunits:

As mentioned earlier, the human glycoprotein hormones hTSH, hFSH, hLH and hCG are composed of non-covalently linked α and β chains (CGA and CGB respectively). The mature α subunit (CGA) is common to all glycoprotein hormones and is comprised of 92 amino acids after removal of a 24 amino acid signal peptide (Morgan, Birken et al. 1975; Fiddes and Goodman 1979). The CGA subunit contains two N-linked oligosaccharides at Asn 52 and Asn 78. N-glycosylation of Asn 52 in CGA is critical for signal transduction as well as biological activity of the hormone (Fralish, Dattilo et al. 2003), possibly because it is located close to the receptor binding site and is important for re-ensuring correct folding of the subunit. The CGB subunit is unique and accounts for biological specificity of each hormone. The peptide structure of hCG was first established by Bahl and colleagues (Bahl, Marz et al. 1974; Bahl 1977). Later, the analysis was confirmed and refined by Furuhashi and colleagues (Furuhashi, Ando et al. 1994). The molecular weight of the main form of hCG heterodimer is 36,000, comprising a 145 amino acid CGB subunit and a 92 amino acid CGA subunit., with the remainder carbohydrate. The oligosaccharide constitutes ~ 25 – 30 % of the molecular weight (~ 10,800). The CGB subunit possesses two N-linked oligosaccharide chains at Asn 13 and Asn 30 and four O-linked oligosaccharide chains at Ser121, Ser127, Ser132 and Ser138 (Fig.7). The molecular weight of CGB subunit is 22 kD of which about one third portion is carbohydrate (Cole 2009). Both CGA and CGB subunits have a similar tertiary

structure, consisting primarily of elongated beta sheets. The two subunits are parallel to each other in the heterodimeric structure (Jameson and Hollenberg 1993; Bousfield, Butnev et al. 1996).

The receptor binding side of hCG has an overall positive charge (Xia, Chen et al. 1994). Residues 44, 88 and 89 of CGA and residues 40 –54 as well as 94 –114 in CGB are important in binding of CG to the LH/CG receptor (Keutmann, Mason et al. 1989; Campbell, Dean-Emig et al. 1991; Chen and Bahl 1992; Keutmann, Hua et al. 1992).

It has been known for a long time that the carbohydrate moieties on the glycoprotein hormones affect their half-life in the circulation and thus the in vivo biological potency of the hormone. Removal of the terminal sialic acid residues markedly reduced the half-life in the circulation (Sairam, 1983). Serial removal of carbohydrates with exoglycosidases indicated a greater loss of biological response than receptor binding (Sairam, 1983). Studies with deglycosylated hormones have been conducted with LH, hCG, FSH and TSH. These studies indicated that removal of N-linked carbohydrate does not interfere with receptor binding but markedly reduces biological responses such as stimulation of adenylate cyclase and steroidogenesis. Removal of carbohydrate from the CGA subunit actually enhances receptor binding when the deglycosylated subunit is recombined with intact CGB subunit. These data indicate that carbohydrate moieties are required on both subunits for full expression of biological activity (Keutmann et al, 1983).

The CGB subunit is believed to have evolved from an ancestral LHB subunit (Talmadge, Boorstein et al. 1984). A single base-pair deletion caused a frame shift in the DNA sequence that encodes 7 amino acids at the 3' end of the open reading frame (ORF)

of *LHB*, which led to the extension of the ORF into the 3' - untranslated region, resulting in a 24 amino acid extension in CGB relative to LHB (Talmadge, Boorstein et al. 1984). There is ~ 82 % sequence identity between first 121 amino acids of CGB and mature LHB (Robert, Pantel et al. 1994). CGB shares 34 % and 37 % sequence identity with FSHB and TSHB respectively (Fiddes and Goodman 1979; Jameson, Albanese et al. 1989).

LH/CG receptor:

The LH/CG receptor belongs to the family of G-protein coupled receptors and consists of a single polypeptide chain with 675 amino acids (Yarney, Sairam et al. 1990; Jia, Oikawa et al. 1991). Both CG and LH specifically bind to the same receptor with similar high affinity and increase the intracellular cyclic AMP (cAMP) levels in the target cells. The receptor consists of two associated functional units: a large extracellular part that specifically recognizes and binds hCG or LH. This part is coupled to a seven transmembrane segment, whose carboxyl terminal associates with the G-protein (Dufau 1998). The intracellular segment plays an active role in signal transduction generated by the binding of hormones to the extracellular domain (Dufau 1998). As mentioned earlier, this G-protein coupled receptor activates the cAMP/PKA mediated signal transduction pathway. Previously it was thought that expression of the LH/CG receptor was restricted to gonadal tissues. However, its presence has been demonstrated in many other tissues than the CL and gonads (Dufau 1998; Licht, Russu et al. 2001; Licht, von Wolff et al. 2003). For example, LH/CG receptor mRNA expression is measurable in human (Lei, Toth et al. 1993; Dufau 1998) as well as porcine (Derecka, Pietila et al. 1995) fallopian

tubes. In mouse, LH/CG receptors were detected in subepithelial cells in the oviduct (Zhang, Shi et al. 2001). LH/CG receptors in myometrium were first identified in pigs (Ziecik, Stanchev et al. 1986), followed by rabbits (Jensen and Odell 1988) and humans (Reshef, Lei et al. 1990). The expression of this receptor in myometrium is higher in the progesterone – dominated phase of the estrous cycle as opposed to the estrogen – dominated phase (Zuo, Lei et al. 1994). Cyclic expression patterns of LH/CG receptor in human menstrual and porcine estrous cycles suggest that estradiol directly up-regulates and progesterone acts through estradiol primed tissue to increase the LH/CG receptor levels in myometrium as well as endometrium (Stepien, Shemesh et al. 1999). LH/CG receptor binding sites in endometrium have been reported in pig (Ziecik, Stanchev et al. 1986), bovine (Freidman, Gurevich et al. 1995), and human. In human, the expression of this receptor was associated with the proliferative and secretory phase of the menstrual cycle (Reshef, Lei et al. 1990).

Together the above results suggest that CG produced by the conceptus might have a local action and that its function extends beyond providing luteotrophic support to the ovary. For example, in baboons CG has multiple effects on the endometrium (Hastings and Fazleabas, 2006; Hastings and Fazleabas, 2003).

Mechanism of eukaryotic transcriptional regulation:

In eukaryotic cells, the protein coding genes are transcribed by RNA polymerase II. In eukaryotes, there are three classes of RNA polymerases: I, II and III. Unlike the sigma factor in eukaryotes that recognize the promoter and unwind the DNA double helix, in eukaryotes, these two major functions are carried out by a set of proteins called

general transcription factors, abbreviated as TFs. The RNA Pol II is associated with six general transcription factors, designated as TF II A, TF II B, TF II D, TF II E, TF II F and TF II H. TFIID consists of TBP (TATA-box binding protein) and TAFs (TBP associated factors). The role of TBP is to bind the core promoter. TAFs may assist TBP in this process. In human cells, TAFs are formed by 12 subunits. One of them, TAF250 (with molecular weight 250 kD), has the histone acetyltransferase activity, which can relieve the binding between DNA and histones in the nucleosome. The transcription factor which catalyzes DNA melting is TFIIH. However, before TFIIH can unwind DNA, the RNA Pol II and at least five general transcription factors (TFIIA is not absolutely necessary) have to form a pre-initiation complex (PIC). First, TF II D including TAFs and TBP, binds to TF II A, which in turn, binds to the TF II B. This complex then binds to RNA Pol II along with TF II F. Formation of this complex is followed by binding of TF II E and TF II H to form the pre-initiation complex (Lee and Young, 1998).

After PIC is assembled at the promoter, TFIIH can use its helicase activity to unwind DNA. This requires energy released from ATP hydrolysis. The DNA melting starts from about -10 bp. Then, RNA Pol II uses nucleoside triphosphates (NTPs) to synthesize a RNA transcript. During RNA elongation, TFIIH remains attached to the RNA polymerase, but all of the other transcription factors have dissociated from PIC. The carboxyl-terminal domain (CTD) of the largest subunit of RNA Pol II is critical for elongation. In the initiation phase, CTD is unphosphorylated, but during elongation it has to be phosphorylated. This domain contains many proline, serine and threonine residues (Lee and Young, 1998).

It has been known for some time that binding of transcriptional activators to the enhancer region, in most cases, is not sufficient to stimulate transcription. Certain co-activators are also required. Similarly, transcriptional repression often requires both repressor binding on the silencer element and the participation of co-repressor proteins. In eukaryotes, the association between DNA and histones prevents access of the polymerase and general transcription factors to the promoter. Histone acetylation catalyzed by HATs can relieve the binding between DNA and histones. Although a subunit of TFIID (TAF250 in human) has the HAT activity, participation of other HATs, such as CBP/P300 can make transcription more efficient (Berger et al, 2002). The following rules apply to most (but not all) cases:

Binding of activators to the enhancer element recruits HATs to relieve association between histones and DNA, thereby enhancing transcription.

Binding of repressors to the silencer element recruits histone deacetylases (denoted by HDs or HDACs) to tighten association between histones and DNA.

The functional link between chromatin structure and transcription activation is the “histone code,” which is generated by methylation or acetylation of specific arginine and lysine residues within histones H3 and H4 (Wang et al, 2001). In fact, the transcriptional apparatus reads this histone code and, as a consequence, activates or represses the neighboring genes. Coordinated methylation, acetylation, and phosphorylation of specific histone residues often promote gene transcription activation. It is now clear that there is a gene-specific and timing-dependent order of events that links chromatin structure modifications and transcription activation (Lo et

al, 2001). Chromatin-modifying enzymes mark histone residues and change nucleosome conformation, allowing the transcriptional machinery to transcribe or repress genes.

DNA binding motifs involved in transcriptional regulation:

One important aspect of transcriptional regulation involves recognition of *cis*-acting DNA sequences by regulatory proteins. X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopic analyses have revealed that most of these regulatory proteins contain a small set of DNA – binding structural motifs that uses their α helices or β sheets to bind to the DNA (Gilbert et al, 1994). In addition to having this sequence-specific DNA-binding domains, transcription factors also contain another domain involved in transactivation of the promoter. Many DNA-binding domains have been well characterized and can be classified according to their general folding and structural characteristics.

Transcriptional regulation of CGA gene:

CGA promoter activity is tightly regulated, with control involving an array of *cis*-acting regulatory elements spanning – 180 to – 60 base pairs upstream of the transcription start (+1) site (Darnell and Boime 1985; Delegeane, Ferland et al. 1987; Jameson, Albanese et al. 1989; Pittman, Clay et al. 1994). This short promoter is just as active as the longest – 1500 bp *CGA* promoter so far tested in choriocarcinoma cells. Early evidence regarding identification of regulatory elements in the *CGA* promoter grew out of the demonstration that choriocarcinoma cell-lines treated with cyclic AMP showed increased CG production. Subsequently, two consensus 18 bp elements spanning 146 to

112 bp upstream of the transcription start site were demonstrated to bind cyclic AMP response element binding proteins (CREB), a basic leucine zipper (bZIP) family of transcription factors. (Delegeane, Ferland et al. 1987; Deutsch, Jameson et al. 1987; Silver, Bokar et al. 1987; Jameson, Albanese et al. 1989). CREB is a substrate for cAMP – dependent protein kinase A (PKA) (Darnell and Boime 1985). In response to cAMP stimulation, CREB becomes phosphorylated by PKA at Ser 133. Mutation of Ser 133 abolishes the ability of CREB to become phosphorylated by PKA (Gonzalez and Montminy 1989). In its non-phosphorylated form, CREB can bind to the CRE, but, only the phosphorylated form can bind CREB – binding protein (CBP) (Kwok, Lundblad et al. 1994). This step appears to be crucial for efficient transactivation of cAMP – responsive genes, including *CGA*. CREB is ubiquitously expressed in mouse, rat and humans (Berkowitz and Gilman 1990). CREB dimerizes through its “leucine” zipper motif and binds to the CRE core sequence (Meyer and Habener 1993). The CRE sites are also known as the “classical enhancer elements”. A single CRE can confer cAMP responsiveness on the *CGA* promoter, but both CREs are required for synergistic activity in both basal as well as cAMP – stimulated transcription (Anderson et al, 1990; Ghosh et al, 2005). Deletion or mutation of one of the CRE sites significantly reduces the *CGA* promoter activity and deletion of both the CRE sites completely abolishes the promoter activity (Andersen, Kennedy et al. 1990). The core sequence, TGACGTCA of the *CGA* CRE has been found in a variety of other promoters whose activities are regulated by cAMP (Montminy, Sevarino et al. 1986; Bokar, Roesler et al. 1988). The CRE also confers responsiveness in some non-trophoblast cell lines such as BHK fibroblasts and PC12 adrenal cells (Delegeane, Ferland et al. 1987; Deutsch, Jameson et al. 1987).

Besides the critical CRE sites, a number of other regulatory regions have been demonstrated to be important for controlling *CGA* promoter activity. One upstream element, spanning -162 / -141 and located distal to the adjacent CRE site, is known as the α -ACT region (Fig. 9). This region binds GATA factors, chiefly GATA 2 and GATA 3 (Steger, Hecht et al. 1994). A highly conserved junctional regulatory element (JRE) spanning (-120/-100) has also been demonstrated to be important (Anderson et al, 1990). This region contains a palindromic site that can bind a 50 kD nuclear factor(s). These factors are distinct from those binding to the upstream regulatory region (URE), CRE or CCAAT box region of the *CGA* promoter. The JRE is identical at nine out of ten bases (ATGGTAATTA) to the consensus octamer – binding transcription factor sequence. The possibility that OCT1 and OCT2 proteins bind to this site was ruled out because OCT1 is ~ 100 kD and OCT2 was ~ 60 kD. Later, it was demonstrated by our laboratory that the JRE binds a POU family transcription factor OCT4, which, when ectopically expressed, significantly represses *CGA* promoter activity (Liu, Leaman et al. 1997). The OCT4 binding site has been inferred to be within the JRE spanning nucleotides – 119 to –112 bp (Fig. 9). Several years later, it was demonstrated that a homeobox family transcription factor, named DLX3 can also occupy a site (- 116 to – 109) within the JRE that overlaps the OCT4-binding site. Ectopic expression of DLX3 modestly up-regulates *CGA* promoter activity (Roberson, Meermann et al. 2001). DLX3 contains a helix – turn – helix DNA binding motif (Alberts et al, 1994).

Recently this laboratory demonstrated that the ETS2 transcription factor could bind to two adjacent ETS-binding sites (EBS) in the proximal part of the promoter (- 82 / - 74) (Fig. 9). ETS2 up-regulation of the promoter is almost entirely dependent upon the

presence of the two CRE and cAMP activation (Ghosh, Sachdev et al, 2005). ETS2 associates with CREB and this interaction is likely to be involved in its regulatory activity (Ghosh, Sachdev et al. 2005) (Fig. 8A, Fig. 8B). ETS2 also has a helix – turn – helix DNA binding motif (Alberts et al, 1994).

The CCAAT box (- 85 / -89) in the *CGA* promoter also plays a critical role in promoter activity in choriocarcinoma cells. Mutation of this element significantly reduces the basal *CGA* promoter activity by ~ 80 % (Kennedy, Andersen et al. 1990). Gel shift analysis demonstrated binding of a CCAAT – enhancer binding protein (CEBP) to the CCAAT sequence on the *CGA* promoter (Kennedy, Andersen et al. 1990). CEBP is a member of bZip DNA binding motif family (Alberts et al, 1994).

A potential AP-1 binding site (-70 TTGATCCCA -62), immediately downstream of two adjacent ETS2 binding sites had also been demonstrated to be crucial for basal as well as ETS2 mediated expression of *CGA* promoter activity (Ghosh, Sachdev et al, 2005).

Transcriptional regulation of *CGB* gene:

In contrast to the *CGA* gene, which is expressed in the pituitary as well as the trophoblast, the *CGB* genes are expressed almost exclusively in the placenta. Much less is known about transcriptional regulation of *CGB* genes than the single *CGA* gene. The *CGB* subunit is represented by seven genes or pseudogenes (Policastro, Daniels-McQueen et al. 1986). Transcriptional regulation of the *CGB* gene has been studied almost exclusively on the *CGB 5* gene, since it and *CGB 3* are the ones predominantly expressed in the placenta and the choriocarcinoma cells. Unlike the *CGA* promoter, the *CGB* promoter lacks a TATA box (Jameson and Lindell 1988). Deletion / mutation

analyses have shown that 78 base pairs upstream of the transcription start site is sufficient for basal transcription (Steger, Buscher et al. 1993). Basal transcription in choriocarcinoma cells is increased with longer promoter constructs, but regions beyond -305 appears to play only a minor role in trophoblast – specific gene expression (Albanese et al, 1991; Pester et al, 1994). The *CGB* promoter up to 305 bp upstream of transcription start site responds positively to cAMP stimulation. Two fragments (-310 / -279) and (-250 / -200) were implicated in cAMP responsiveness (Steger, Buscher et al. 1993) (Fig. 10), but neither contains a consensus CRE motif (TGACGTCA). Gel shift analysis also failed to show any CREB binding (Albanese, Kay et al. 1991). Two Sp1/Activating protein (AP) – 2 binding proteins have been implicated in basal *CGB* activity as well as cAMP responsiveness (Johnson and Jameson 2000).

Like *CGA*, the POU domain transcription factor OCT4 binds to an octamer element around – 270 on the *CGB* promoter. OCT4 represses the *CGB* promoter containing the OCT – binding site by ~ 90 %, and its deletion reverses this effect, suggesting that silencing of the *CGB* promoter by OCT4 depends on its binding ability. As mentioned earlier, unlike *CGB*, silencing of the *CGA* promoter by OCT4 is independent of its binding ability.

ETS2, a member of an extensive family of proteins that resemble the *v-ets* oncogene in the E26 retrovirus (Crepieux, Coll et al. 1994) has an apparently major role in controlling *CGB* promoter activity (Ghosh, Ezashi et al. 2003). Two ETS2 binding sites in the proximal part of the *CGB* promoter have been found (Fig. 10). Ectopically-expressed ETS2 modestly up-regulated *CGB* promoter activity, but this effect was markedly enhanced ~100-fold in presence of Ras/MAPK. Although, a consensus CRE

motif is lacking in *CGB* promoter, addition of 8 – bromo – cAMP with ectopically expressed ETS2 also dramatically up-regulated the *CGB* promoter activity (Ghosh, Ezashi et al. 2003).

Description of some of the key transcription factors involved in regulation of *CGA* promoter activity:

ETS2 belongs to the family of ETS/E26 family of transcription factors (Watson, McWilliams et al. 1988), which is comprised of at least 26 unique family members. All the members share an 85 amino acids ETS domain (DNA binding motif). The protein also possesses an approximately 100 amino acids POINTED domain that is highly conserved within the family (Tymms and Kola 1994) (Fig. 9A). ETS2 regulates a variety of genes expressed in trophoblast (see below) and is also known to be important for differentiation of the mouse placenta (Yamamoto, Flannery et al. 1998). ETS2 is a key transcriptional regulator of the *IFN- τ* genes (*IFNT*) in cattle and sheep (Ezashi, Ealy et al. 1998), the *CGA* and *CGB* subunit genes (Johnson and Jameson 2000; Ghosh, Ezashi et al. 2003; Ghosh, Sachdev et al. 2005), and *CYP11* (P-450 side-chain cleavage enzyme) genes in human (Pestell, Albanese et al. 1996), and the *PL-II* gene in the mouse (Sun and Duckworth 1999). Moreover, urokinase-type plasminogen activator (*PLAU*) and several metalloproteinases, including MMP9, implicated in implantation and trophoblast invasion are also regulated by ETS2, suggesting its potential role or involvement in invasive placentation (Stacey, Fowles et al. 1995; Watabe, Yoshida et al. 1998).

ETS proteins bind to purine-rich sequences with a core motif of GGAA/T. Two overlapping Ets2-binding sites in the proximal part of the *CGA* promoter have been

previously identified in our laboratory (EBS1, located -81 to -78; EBS2, located -75 to -72), (Ghosh, Sachdev et al. 2005) (Fig. 11A). The association of ETS2 with these sequences has been demonstrated by electrophoretic mobility shift assays (EMSA) and, in vivo, by chromatin immunoprecipitation (ChIP) assays (Ghosh, Sachdev et al. 2005). Transient transfection assays performed on JAr and JEG choriocarcinoma cell-lines have shown that ETS2 up-regulates the *CGA* promoter activity only modestly (~3.5 to 4-fold) and that a single bp mutation in either or both of the ETS2 binding sites inhibits this effect. Ectopic expression of ETS2 also increases the steady-state levels of phosphorylated CREB, suggesting a potential role of cross talk between PKA – mediated signaling and ETS2 - mediated transactivation in regulation of *CGA* promoter activity (Ghosh, Sachdev et al, 2005). In a parallel study from our laboratory, it was demonstrated that ETS2 over-expression in JAr choriocarcinoma cells transactivated the *boIFNT* promoter by ~ 20 fold. Moreover, this transactivation was repressed in a dose-dependent manner by POU domain transcription factor, OCT4 (Ezashi, Ghosh et al. 2001). These results suggest considerable similarities between control of expression of the *IFNT* and *CGB* genes.

DLX3 is one of six genes belonging to the homeobox family related to that of *Drosophila* Distal-less (Dll) group. DLX3 is required for normal placental development (Morasso, Grinberg et al. 1999). In mammals, expression of the DLX3 gene is largely restricted to branchial arches, dental tissues, epithelial derivatives and the placenta (Bendall and Abate-Shen 2000). DLX3 and DLX4 expression patterns slightly overlap in mammalian placenta (Quinn, Johnson et al. 1997; Quinn, Kilpatrick et al. 1998). In the mouse, DLX3 expression within placenta is restricted to labyrinthine trophoblast layer,

ectoplacental cone and chorionic plate (Morasso, Grinberg et al. 1999). It appears to have a role in controlling *CGA* expression, although the up-regulation observed was relatively small (Roberson, Meermann et al. 2001). *DLX3* is localized in the trophoblast layer of the human chorionic villus and binds to the *CGA* promoter in the junctional regulatory region (Roberson, Meermann et al. 2001). The *DLX3* binding site overlaps the binding site of *OCT4* (Fig. 11B).

OCT4 belongs to the POU (pit-oct-unc) transcription factor family. *OCT4* is expressed in all cells of cleavage stage embryos of mouse, pig and human (Palmieri, Peter et al. 1994) and also in the early stages of trophoctoderm formation, although it is down-regulated in the mouse at about the time of hatching from the zona pellucida (Palmieri et al, 1994). It is also expressed in the early trophoctoderm of porcine, bovine and human blastocysts (Palmieri et al, 1994). Although not proven, the up-regulation of genes silenced by *OCT4* would presumably coincide with the down-regulation of this transcription factor (Ezashi et al, 2001). *OCT4* expression in the mouse and possibly the human becomes confined exclusively to the inner cell mass (ICM) of the expanded blastocyst and later to the epiblast prior to differentiation of the three main germ layers.

OCT4 consists of a POU domain, which is comprised of two sub-domains: a 75 amino acid N terminal POU specific (POUs) and a 60 amino acid C terminal homeodomain (POUh) (Pan, Chang et al. 2002) (Fig. 11C). These domains are highly conserved among all members of the family and are also connected via a non-conserved spacer sequence comprised of 15 – 25 amino acids (Pan et al, 2002). So far, POU domain proteins have been cloned in various species, including *Drosophila*, *Xenopus*, Zebrafish, Chicken, Mouse and Human. They are classified into five groups based on their primary

sequence similarities in the POU domain (Rosenfeld, 1991). Some POU proteins, such as Pit1 in mouse and Unc86 in *C. elegans* play a significant role in developmental regulation (Li et al, 1990; Finney and Ruvkun, 1990). Pit1 binds and activates prolactin and TSH-beta subunit genes (Anderson and Rosenfeld, 1994).

OCT4, when ectopically expressed in JAr cells repressed the promoter activities of both the *CGA* and *CGB* subunit genes by ~ 90 % (Liu and Roberts 1996; Liu, Leaman et al. 1997). Although OCT4 binding to the *CGB* promoter was associated with silencing, repression of the *CGA* promoter by OCT4 appeared to be independent of its binding ability. As mentioned earlier, OCT4 binds to an octamer sequence (- 119 AAATGGTAAT – 112) in the JRE of the *CGA* promoter (Liu, Leaman et al, 1997). Stable transfection of JAr choriocarcinoma cells with OCT4 expression vector reduced the endogenous *CGA* mRNA as well as protein levels by 70 – 80 %. Moreover, radioimmuno assay of proteins secreted into the medium by these stably transfected JAr cells showed significant reduction of *CGA* production, suggesting the role of OCT4 as a potent negative regulator of *CGA* transcriptional activity (Liu, Leaman et al, 1997). POU domain transcription factors including OCT1 have been previously demonstrated to regulate transcription through their cooperative interactions with other transcription factors (Verrijzer, Alkema et al. 1992; Verrijzer, Strating et al. 1992; Verrijzer, van Oosterhout et al. 1992; Gstaiger, Knoepfel et al. 1995; Luo and Roeder 1995; Strubin, Newell et al. 1995). OCT4 silences *boIFNT* promoter activity by interfering with ETS2 – mediated transactivation of the promoter (Ezashi et al, 2001). The work of Liu & Roberts (Liu and Roberts et al, 1996; Liu, Leaman et al, 1997) with *CGA* and *CGB* and of Ezashi & Roberts (Ezashi et al, 2001) on the *IFNT* promoter strongly suggested that OCT4

mediates silencing of the *CGA* promoter either by interacting with transcriptional activators bound to the promoter, thereby preventing transactivation (quenching mechanism), or by interacting with crucial transactivators before they became associated with the promoter (squenching mechanism). However, these possibilities have not been distinguished. It would be interesting to explore the type of mechanisms underlying the silencing activity of OCT4. It would also be interesting to identify genes other than *CGA* or *CGB*, secreted from the trophoblast that are being differentially regulated by OCT4.

Molecular framework underlying pluripotency and self-renewal:

Potential use of pluripotent stem cells as a source of differentiated cells for repair of degenerating or damaged tissues in humans has enormous medical potential, but has also caused controversy because the human cells are derived from embryos. Embryonic stem cells, isolated from early mouse and human embryos, for example, can be directed to differentiate into a wide range, possibly all cell types (Wobus, 2001).

Another kind of stem cell that has been derived from both outgrowths of trophectoderm and the ectoplacental cone of mouse blastocysts is the trophoblast stem cell (TSC) (Rossant, 2007). However, a similar success has not been achieved with other species, including the human. ESC and TSC use distinct signaling pathways to maintain cell proliferation and also require different sets of transcription factors for specification of different cell fates and lineages (Rossant, 2007; Kunath et al, 2004).

OCT4 expression is believed to be essential for maintenance of pluripotency and “stemness” in ESCs. A critical level of OCT4 expression is required for maintenance of stem cell characteristics. This transcription factor acts as an important molecular switch

and alteration in the level of expression of OCT4 alters cell fate (Niwa et al, 2000). An increase in OCT4 expression causes differentiation towards primitive endoderm and mesoderm. On the contrary, a decrease in expression induces loss of pluripotency and drives differentiation towards trophoctoderm (Niwa 2000; Niwa 2000; Niwa, Miyazaki et al. 2000). These observations suggest that OCT4 may have a unique function in pluripotent cells to as a binary “on-off” switch for certain genes involved in lineage specification. In some cases, OCT4 can act as a repressor of target genes whereas in other cases, it acts as an activator (Niwa, 2000).

While trophoctoderm contributes exclusively to the trophoblast (Rossant, 1995), the ICM and its derivative, the epiblast, give rise to the embryo proper as well as extraembryonic endoderm, yolk sac, and the allantois and amnion. OCT4 is initially expressed in both the ICM and trophoctoderm at the blastocyst stage, but expression is quickly lost from the latter but retained in the ICM and in the early epiblast. Eventually, it becomes confined to the developing germ cells (Scholer, Dressler et al. 1990; Palmieri, Peter et al. 1994). Another transcription factor, SOX2 exhibits a similar expression pattern to OCT4 during early stages of mouse embryo development, but, unlike OCT4, SOX2 continues to be expressed in the trophoctoderm. SOX2 is probably important for lineage specification and its deletion causes early post implantation failure (Roberts, Ezashi et al. 2004). SOX2, in association with OCT4, regulates the production of FGF4, a necessary growth factor for proliferation of trophoctoderm (Rossant and Cross 2001; Rossant, Chazaud et al. 2003).

NANOG is a third transcription factor that is considered essential for maintaining pluripotency and in regulating fate of ICM / epiblast cells as development proceeds. For

example, mouse embryos lacking NANOG fail to develop beyond the blastocyst stage due to lack of epiblast (Mitsui, Tokuzawa et al. 2003). One other function is to prevent differentiation of ICM/epiblast to primitive endoderm (Chambers, Colby et al. 2003). A composite OCT4/SOX2 binding motif in the *NANOG* promoter regulates *NANOG* gene expression (Kuroda, Tada et al. 2005; Rodda, Chew et al. 2005). EMSA and ChIP have demonstrated direct binding of OCT4 and SOX2 to this promoter sequence (Rodda, Chew et al. 2005). These findings suggest a significant role of OCT4 and SOX2 in regulating *NANOG* expression and, hence, maintaining the pluripotent state of both ESC and ICM/epiblast. Additional studies involving ChIP coupled with DNA microarray (ChIP – on Chip) analyses have identified DNA binding regions for OCT4, SOX2 and *NANOG* on large numbers of genes in human ESC (Boyer, Lee et al. 2005). At least half of the genes that bound OCT4 to their promoter regions also bound SOX2, and the majority of these genes also had associated *NANOG* as well, implicating the importance of a strong inter – regulatory network of these three transcription factors in maintaining ES cell pluripotency and self-renewal (Loh, Wu et al. 2006; Babaie, Herwig et al. 2007). The role of this association is probably in silencing transcription factors whose expression would drive differentiation along specific lineages. In addition, quantitative chromatin immunoprecipitation analysis indicated that chromatin remodeling takes place on OCT4 and *NANOG* to establish a conformation that is compatible with transcriptional activation (Freberg et al, 2007). Based on these large-scale data sets, it was proposed that OCT4, SOX2 and *NANOG* collaborate to form a complex regulatory circuitry that contributes towards self-renewal and pluripotency (Boyer et al, 2005).

Apart from OCT4, SOX2 and NANOG, several other genes are characteristically up-regulated in pluripotent stem cells compared to normal somatic cells. Some of these genes are also often up-regulated in tumors. Examples include STAT3 (Niwa, Burdon et al. 1998), E-RAS (Takahashi, Mitsui et al. 2003), c-MYC (Cartwright, McLean et al. 2005) and KLF4 (Li, McClintick et al. 2005). These genes probably contribute towards pluripotency and/or the long-term maintenance of the ES cell phenotype as well as proliferative capacity of ES cells in culture.

A number of extrinsic growth factors have been reported to play roles in maintenance of pluripotency in ESCs. Leukemia inhibitory factor (LIF) supports the undifferentiated state of mouse ESCs by activating the signal transducer STAT3 (Smith et al, 1988). LIF withdrawal or inhibition of STAT3 drives differentiation of mouse ESCs towards a morphologically mixed population of cells (Niwa, Burdon et al. 1998). Another extrinsic factor known to support self-renewal in mouse ESCs is BMP4. In the presence of LIF, BMP4 can enhance self-renewal and pluripotency in ESCs by activating members of the Id gene (Inhibition of differentiation) family (Ying, Nichols et al. 2003). Unlike in mouse ESCs, BMP4 causes rapid differentiation in human ESCs and LIF is also not sufficient to maintain human ESC self-renewal (Daheron, Opitz et al. 2004). Apart from LIF and BMP4, the Wnt pathway also delays the differentiation of mouse and human ESCs (Sato, Meijer et al. 2004). Although OCT4 is known as a key factor in maintenance and self-renewal of ESCs, it is not the only master regulator. On withdrawal of LIF, OCT4 alone is incapable of preventing mouse ESC differentiation, suggesting the necessity of additional factors in regulating pluripotency in ESCs.

Reprogramming of somatic cell nuclei by transfer into oocytes was first demonstrated in sheep (Wilmut et al., 1997) and since confirmed in a wide range of species (with the notable exception of the human) (Schnieke, Kind et al. 1997). Another approach has been to fuse somatic cells with ES cells (Cowan, Atienza et al. 2005). Such studies demonstrate that pluripotency can be restored in a terminally differentiated cell and suggest that some factors present in the oocyte or ES cells can reprogram a somatic nucleus to one that has pluripotent properties.

A very exciting recent strategy has been to reprogram somatic cells by expressing combinations of “stemness” genes. In the initial report, murine fibroblasts were infected with integrating retroviral-based vectors that drove expression of four factors, OCT4, SOX2, KLF4 and c-MYC (Takahashi and Yamanaka 2006). The resulting cells were quite similar in phenotype to murine ES cells and were shown to be pluripotent by a range of criteria, including the ability to contribute to chimeras and generate pups. Later, analogous procedures were employed to reprogram human fibroblast to a pluripotent state by using the same or different combination of “stemness” genes (Takahashi, Tanabe et al. 2007; Huangfu, Osafune et al. 2008). The efficiency of reprogramming has consistently been low, but has been improved by adding other ectopically expressed transcription factors to the mix or by exposing the cells during re-programming to low molecular weight compounds, such as inhibition of DNA methyltransferase or HDAC inhibitors (Huangfu, Maehr et al. 2008; Huangfu, Osafune et al. 2008). These studies also suggested that chromatin remodeling is a rate – limiting step in the reprogramming process (Huangfu, Maehr et al. 2008). Over the past few months, somatic cells have been

reprogrammed with vectors that do not integrate, vectors that can be removed after re-programming is complete, and by transfecting with the proteins themselves.

There is interest in determining which of the re-programming genes is most essential and their individual roles in reprogramming. *OCT4* has been used in all the re-programming gene combinations employed to date. Some data have suggested that *KLF4* is essential for reprogramming of mouse cells to a pluripotent state (Eminli, Utikal et al. 2008; Kim, Chu et al. 2008; Kim, Zaehres et al. 2008; Shi, Desponts et al. 2008), but experiments with human fibroblasts have indicated that re-programming can be achieved with only *OCT4* and *SOX2*, without making use of *KLF4* (Huangfu, Osafune et al. 2008). Retrovirally transduced c-MYC provoked tumorigenicity that rendered iPS cells unsuitable for transplantation (Okita et al, 2007). Introduction of *OCT4*, *SOX2* and *KLF4* successfully demonstrated reprogramming iPS cells from mouse and human fibroblast (Nakagawa et al, 2008). Yu et al took this study one step further by reprogramming human somatic cells in the absence of *KLF4*. Replacement of *KLF4* and c-MYC by *NANOG* and *LIN28* also made *KLF4* dispensable for reprogramming (Yu et al, 2007).

One goal of some research is to dispense with using genes altogether and to focus on using either the proteins themselves to drive re-programming (Takahashi and Yamanaka, 2006) or to use a purely chemical approach. What is clear is that if induced pluripotent stem cells are to be used to create “individualized” stem cell lines for therapy in human patients, these cells should have any foreign, integrated gene and vector sequences removed.

This research described in this thesis has been directed towards understanding the transcriptional control mechanisms operating on the expression of chorionic

gonadotropin (CG), a hormone crucial for the survival of the human conceptus. The emphasis is particularly on processes likely to be involved in the early up-regulation of expression that occurs in early pregnancy when there is a “race” between the forces that lead to luteolysis and the initiation of a new ovulatory cycle and ones emanating from the conceptus that lead to luteal rescue. The work in Chapter II is focused on the *CGA* gene and on the role of two transcription factors that up-regulate its expression (ETS2 and DLX3) and one that represses it (OCT4). In Chapter III, the research has taken advantage of JAr cell lines developed well over a decade ago that stably express OCT4 and have a greatly diminished ability to express both CG subunit genes and to produce the two subunit proteins (Liu and Roberts et al, 1996; Liu, Leaman et al, 1997). The goal here was to determine which other genes in these cells are down-regulated when OCT4 is expressed and whether there is some degree of re-programming of these cells to a less differentiated state.

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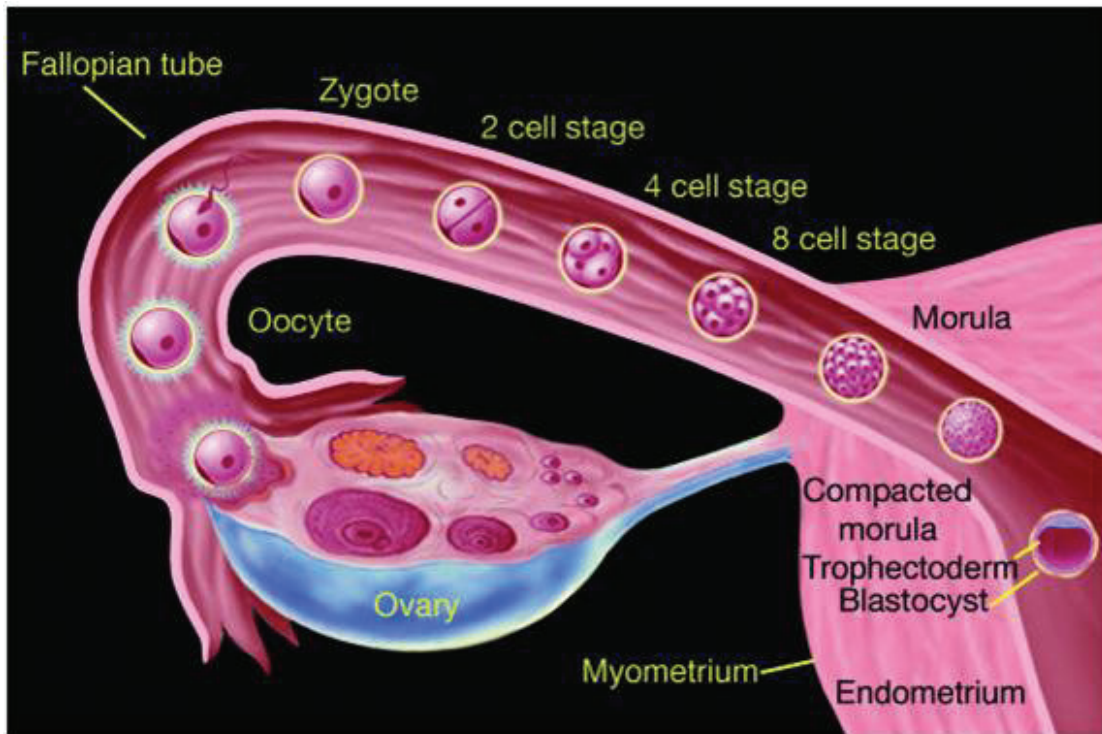
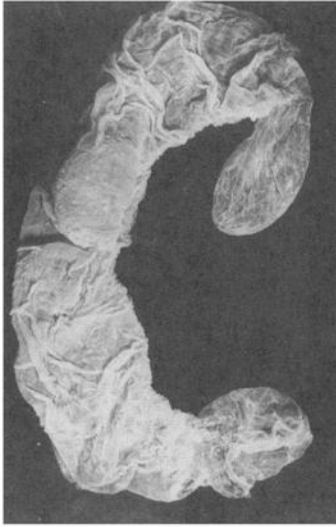
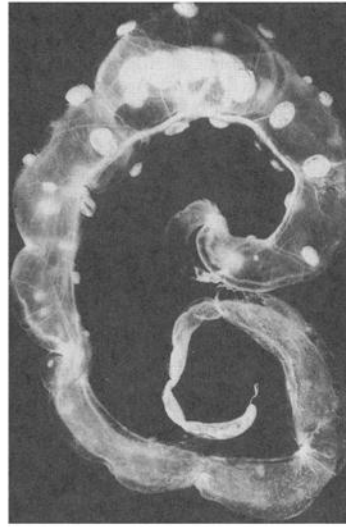


Fig. 1: Schematic representation of early stages of human development starting from fertilization to blastocyst formation (Red-Horse et al., 2004)

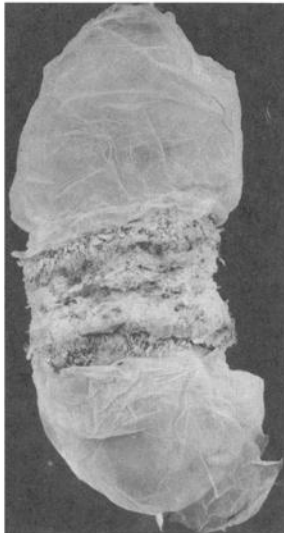
Diffuse



Cotyledonary



Zonary



Discoid

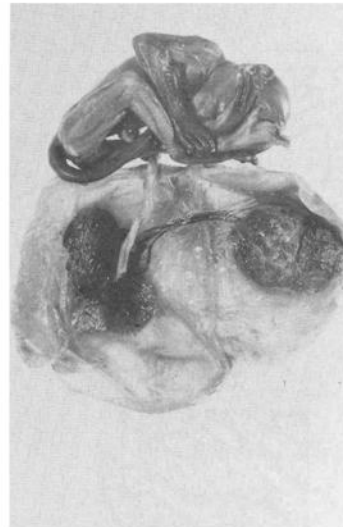


Fig.2. Classification of four major placental types based on their shape of vascularized fetal membranes in contact with maternal tissues. The categories include diffuse placenta (found in pigs and horses), cotyledonary placenta (found in cattle and sheep), zonary placenta (found in dogs and cats) and discoid placenta (found in mouse and higher primates, including humans (Telugu et al, 2007).

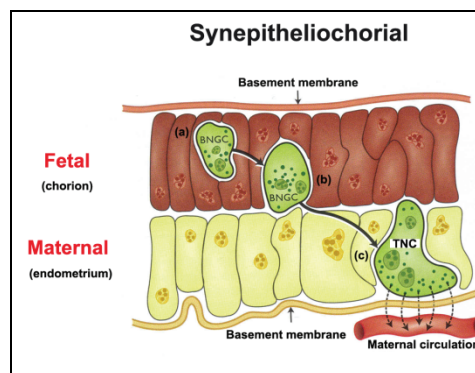
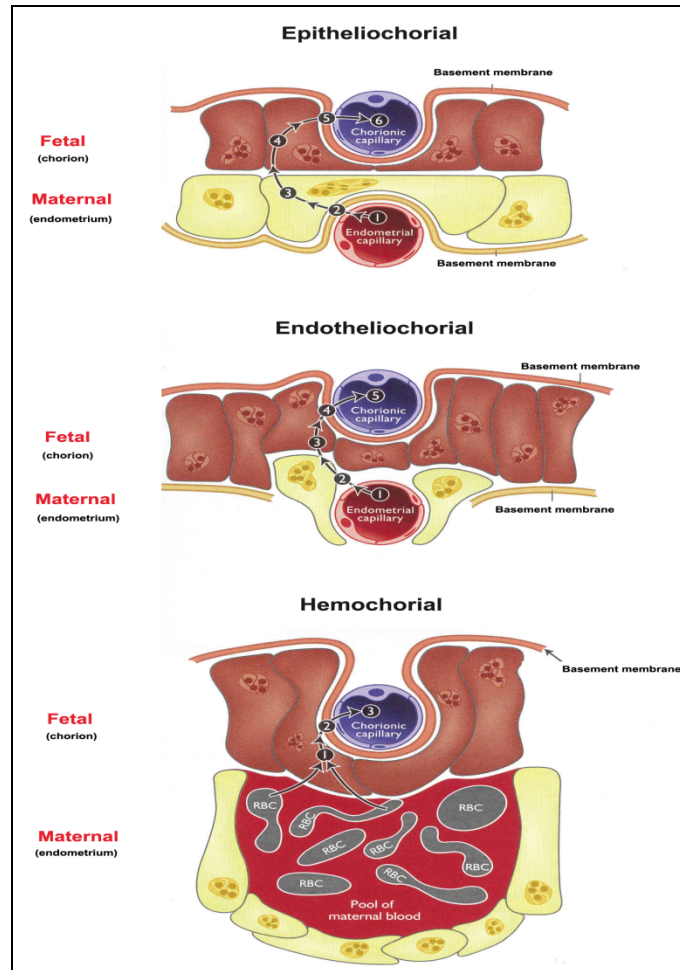


Fig.3. Placental classification based on the number of intact cell layers at the maternal-fetal interface. In epitheliochorial placenta, six tissue types separate maternal and fetal blood. In endotheliochorial type, there are four tissue layers. Synepitheliochorial type is derived from epitheliochorial placenta and has specialized trophoblast population of binucleate giant cells. The hemochorial type has only three tissues, fetal trophoblast, fetal connective tissue and fetal endothelial cells (Telugu et al, 2007).

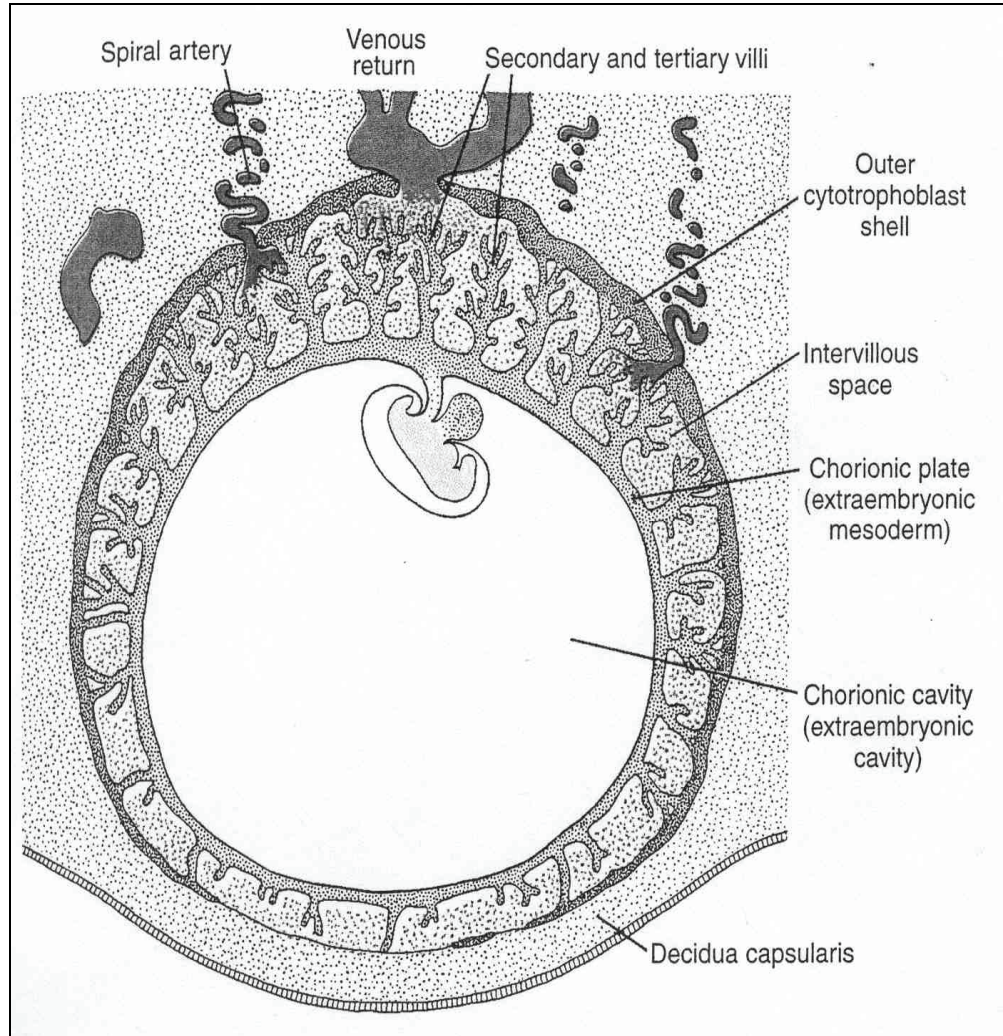


Fig.4. A schematic representation of hemochorial placentation in humans at the second month of development. The embryonic pole consists of numerous chorionic villi. On the contrary, the abembryonic pole fewer villi. Proliferation of cytotrophoblast cells through the syncytiotrophoblast cells leads to formation of the villi. The cytotrophoblasts finally penetrate the syncytiotrophoblasts to form the anchoring villi (Telugu et al, 2007).

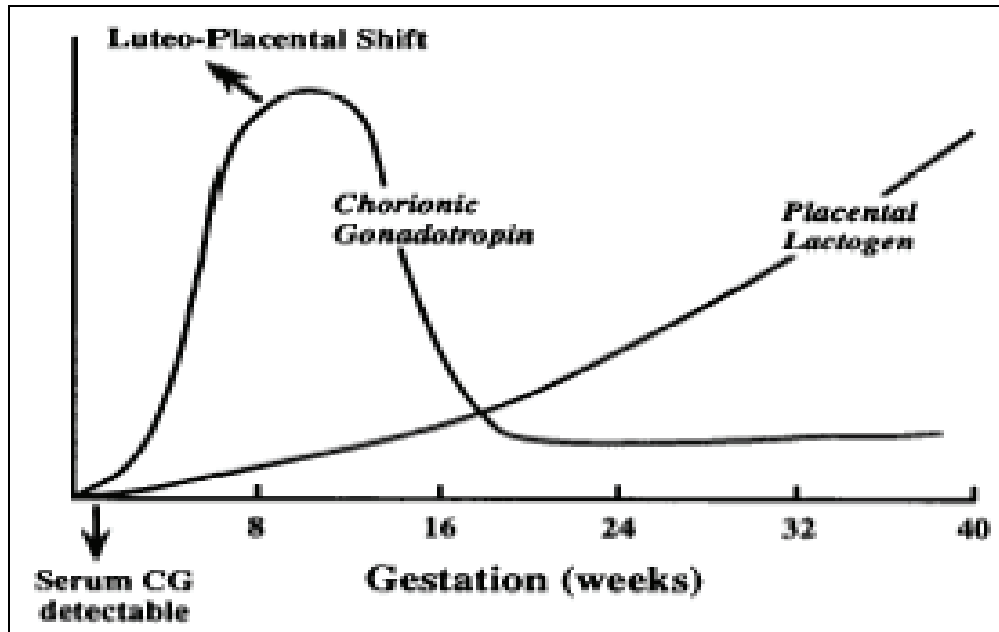


Fig.5. Secretion of CG during pregnancy. (Jameson and Hollenberg, 1993)

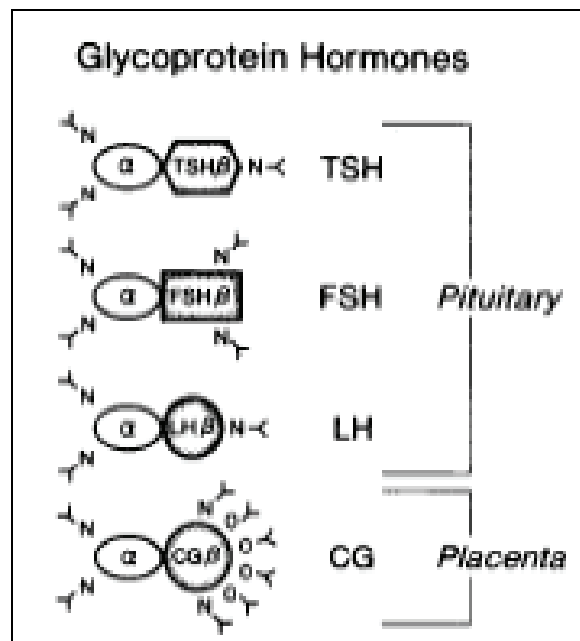


Fig. 6. Structure of glycoprotein hormones with common α -subunit and unique β -subunits. (Jameson and Hollenberg, 1993)

α -subunit, 92 amino acids

ala-pro-asp-val-gln-asp-cys-pro-glu-cys-thr-leu-gln-glu-asp-pro-phe-phe-ser-gln-pro-gly-ala-pro-ile-leu-gln-cys-met-gly-
 1 N
 cys-cys-phe-ser-arg-ala-tyr-pro-thr-pro-leu-arg-ser-lys-lys-thr-met-leu-val-gln-lys-asn-val-thr-ser-glu-ser-thr-cys-cys-
 31 N 52
 val-ala-lys-ser-tyr-asn-arg-val-thr-val-met-gly-gly-phe-lys-val-glu-asn-his-thr-ala-cys-his-cys-ser-thr-cys-tyr-tyr-his-lys-ser
 61 78 92

β -subunit, 145 amino acids

ser-lys-glu-pro-leu-arg-pro-arg-cys-arg-pro-ile-asn-ala-thr-leu-ala-val-glu-lys-glu-gly-cys-pro-val-cys-ile-thr-val-asn-
 1 N N
13 30
 thr-thr-ile-cys-ala-gly-tyr-cys-pro-thr-met-thr-arg-val-leu-gln-gly-val-leu-pro-ala-leu-pro-gln-val-val-cys-asn-tyr-arg-
 31
 asp-val-arg-phe-glu-ser-ile-arg-leu-pro-gly-cys-pro-arg-gly-val-asn-pro-val-val-ser-tyr-ala-val-ala-leu-ser-cys-gln-cys-
 61
 ala-leu-cys-arg-arg-ser-thr-thr-asp-cys-gly-gly-pro-lys-asp-his-pro-leu-thr-cys-asp-asp-pro-arg-phe-gln-asp-
 91 O O O O
 ser-ser-ser-ser-lys-ala-pro-pro-pro-ser-leu-pro-ser-pro-ser-arg-leu-pro-gly-pro-ser-asp-thr-pro-ile-leu-pro-gln
 118 121 127 132 138 145

Fig. 7. Amino acid sequence of the human *CGA* and *CGB* subunits (Cole et al, 2009). The numbers indicate the positions of amino acid residues and N and O indicate the positions of N and O – linked oligosaccharides. These amino acids comprise the mature chain after removal of the signal sequence.

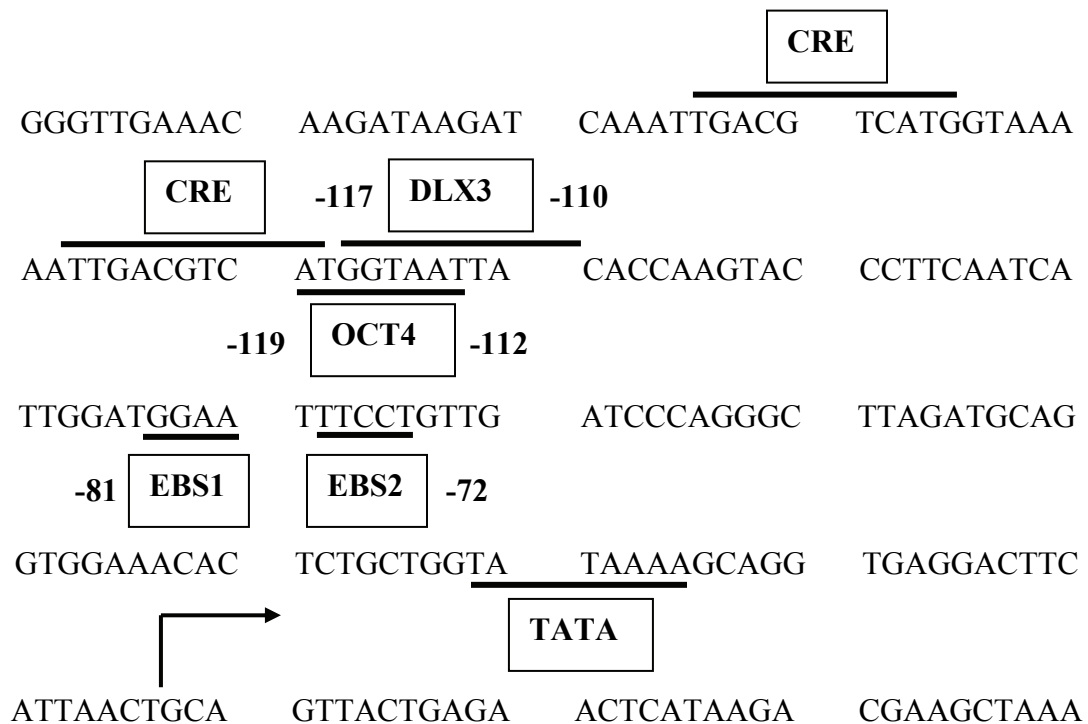


Fig.8. Human *CGA* promoter sequence showing two adjacent CRE sites in the upstream regulatory region. OCT4 and DLX3 binding sites in the junctional regulatory region of the promoter overlap each other. Further downstream in the more proximal region, there are two adjacent ETS2 binding sites (EBS 1 and 2). The arrow indicates the transcription start (+1) (G) site. The numbers indicate the position of the binding sequences.

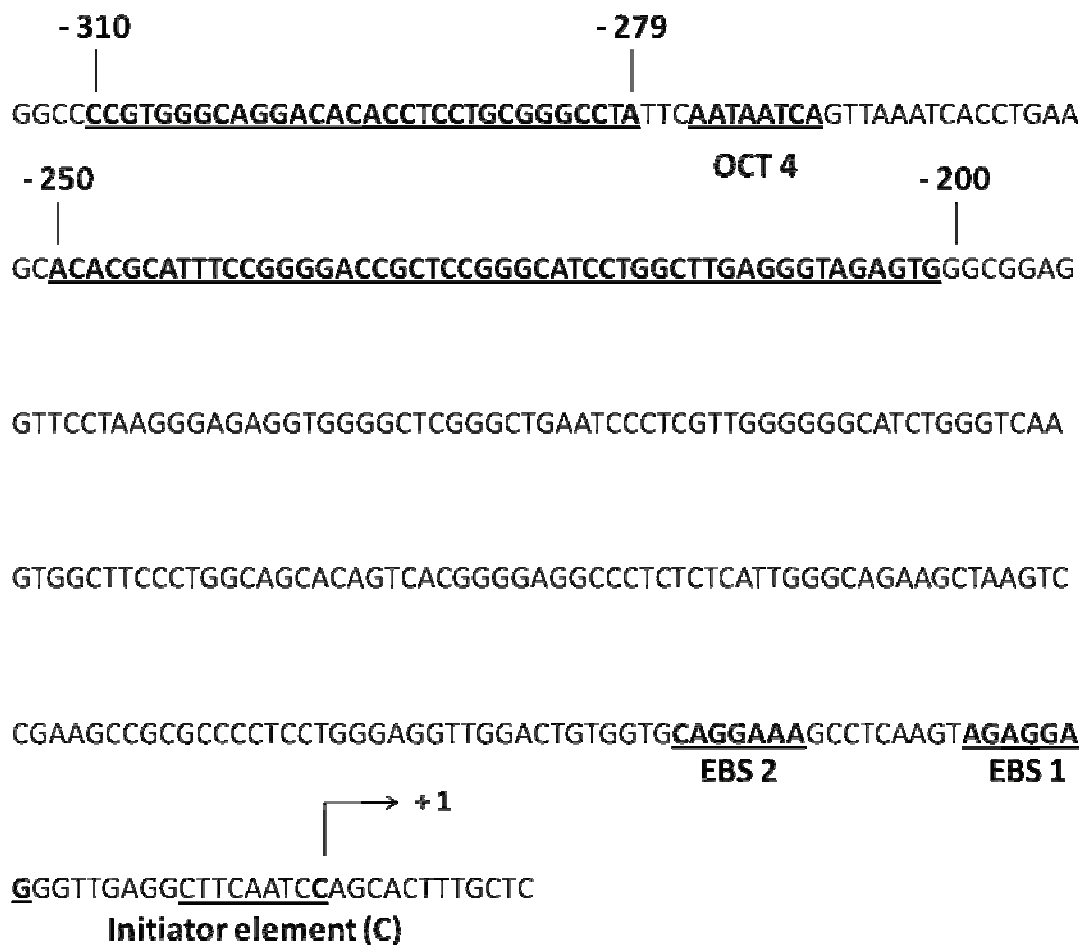


Fig.9. Human *CGB5* promoter sequence showing two ETS binding sequences (EBS 1 and EBS 2) in the proximal part. Transcription start site (+ 1) (C) is indicated by arrow. The *CGB* promoter is TATA less but instead, have a possible initiator element (C). Two underlined regions (- 310 to – 279) and (- 250 to – 200) has been implicated in cAMP responsiveness. OCT4 transcription factor binds to an octamer element ~ 270 bp upstream of transcription start site.

Fig. 10. (A). Schematic diagram of ETS2 transcription factor showing its pointer (PNT) and ETS binding (ETS) domain.

(B). *CGA* promoter showing two adjacent ETS2 binding domains spanning from – 82 to – 74 bp.

(C). *CGA* promoter showing DLX3 binding site. DLX3 binds to an octamer sequence (-117 to – 110) in the upstream JRE.

(D). *CGA* promoter showing OCT4 binding site. OCT4 binds to an octamer element (-119 to -112) in the upstream JRE. This sequence overlaps the DLX3 binding site.

(E). Schematic representation showing domains of OCT4 protein. N-terminal is the transactivation domain and POU domain is the DNA binding domain with a POU-specific and POU-homeo sub-domains.

Chapter II

Relative roles of OCT4, ETS2, and DLX3 in the regulation of CGA gene promoter activity.

Abstract:

OCT4, a key transcription factor in maintaining pluripotency in early embryos and embryonic stem cells, strongly represses expression of trophoblast-specific genes including bovine interferon tau (*bIFNT*) and the subunit genes (*CGA* & *CGB*) of human chorionic gonadotrophin (HCG). Here, we have investigated the mechanism underlying OCT4-mediated repression of *CGA*, concentrating on the interaction between OCT4 and ETS2, a transcriptional activator of many signature genes of trophoblast, including *CGA*. ETS2 binds to two overlapping sequences in the proximal part of the *CGA* promoter and modestly up-regulates its activity. This transactivation is reversed in a dose-dependent manner by co-expression of OCT4 in JAr choriocarcinoma cells. Mutation of the OCT4 binding site in the junctional regulatory region (JRE) of the *CGA* promoter did not alter the ability of OCT4 to act as a repressor, suggesting that its association with DNA was unimportant for its silencing activity. OCT4 required both its N-terminal and POU domains for full repressive activity; neither used alone was an effective silencer. An ETS2-DNA complex was only formed in absence of OCT4 expression, suggesting that OCT4 prevents ETS2 from binding to the *CGA* promoter, presumably by squelching ETS2-mediated transactivation. Although an intact octamer DNA sequence was not important for OCT4 silencing, mutations in this sequence led to a significant drop in basal promoter activity, suggesting the importance of the element in binding an additional transactivating transcription factor. As the Hox gene family member, DLX3 binds close

to the octamer element and modestly up-regulates the basal activity of the *CGA* promoter, its transactivational properties in combination with ETS2 were examined further. ETS2 and DLX3, which are both expressed at low levels in JAr cells, when over-expressed together synergistically up-regulate the *CGA* promoter activity in a dose-dependent manner up to 1000-fold. Co-immunoprecipitation analysis has revealed that the two transcription factors form a complex and that both must bind to the promoter for the combination to be effective. This physical interaction was compromised by the presence of OCT4. Together the data show that the presence of even low concentrations of OCT4 is likely to prevent expression of *CGA* through its ability to squelch ETS2 action and disrupt the synergistic effects of ETS2 with DLX3.

Introduction:

Chorionic gonadotrophin (CG) is considered as the primary signal for maternal recognition of pregnancy in higher primates, including humans (Roberts, Xie et al. 1996). It acts as a luteotrophic hormone and maintains progesterone secretion from the corpus luteum (CL) and thereby prevents the latter from the functional loss of activity that would normally occur at the end of an ovarian cycle in which a pregnancy was not initiated (Duncan 2000). Human CG (hCG) is first expressed from the trophoblast cells of the pre-implantation embryo, beginning about day 7 or 8 post-fertilization during the onset of hatching and implantation to the uterine wall (Lopata and Oliva 1993; Lopata 1996; Afshar, Stanculescu et al. 2007). After implantation, the concentrations of hCG, which is generally measured by immune assays that recognize the β subunit, rise exponentially with an average doubling time of 31h (Pittaway, Reish et al. 1985). Subsequently

production of the intact hormone falls off, although circulating concentrations of the free α -subunit remain high. This observation has two important implications. First it suggests that *CGA* might have its own role during pregnancy; second it appears likely that the expression of the two subunit genes might not be coordinated.

As indicated above, CG is a heterodimer, consisting of an α - and a β -subunit. The former is common to all glycoprotein hormones including luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) (Fig.5 in chapter I). The β -subunits of each of these three hormones are unique and account for their receptor binding and biological specificities (Jameson and Hollenberg 1993; Bousfield, Butnev et al. 1996).

The transcriptional control mechanisms responsible for *hCG* subunit expression have been investigated extensively, and a number of key *cis*-regulatory elements have been identified in each of the genes. Various combinations of transcription factors differentially regulate the *CGA* promoter activity by binding to these gene regions (Fig. 1). Two adjacent 18 bp repeat elements, known as cyclic AMP (cAMP) response elements (CRE) spanning -147 to -111 bp are crucial regulatory elements (Andersen, Kennedy et al. 1990; Nilson, Bokar et al. 1991) and bind a phosphorylated form of CRE-binding protein (CREB) (Meyer and Habener 1993; Meyer, Waeber et al. 1993). An α -ACT region spanning -162 to -141 binds GATA family members, most likely GATA2 (Steger, Altschmied et al. 1991; Steger, Buscher et al. 1993; Steger, Hecht et al. 1994; Hardingham, Chawla et al. 1997). Another potential transactivating transcription factor, AP-2 γ , binds immediately upstream of the GATA binding site (Steger, Hecht et al. 1994; LiCalsi, Christophe et al. 2000). In various combinations, ectopic over-expression of

these transcription factors can up-regulate reporter gene expression from the *CGA* promoter in choriocarcinoma cells.

Relatively recently, this laboratory demonstrated that the *CGA* promoter contains a pair of overlapping ETS2-binding elements spanning the -82 to -74 region upstream of the transcription start site (Ghosh, Sachdev et al. 2005). Importantly, ETS2 functioned combinatorially with the cAMP signal transduction pathway to activate reporter gene expression driven by the *CGA* promoter. In addition, mutation of the ETS2-binding sites dramatically reduced up-regulation by PKA, and mutations within the two CREs abolished responsiveness to ETS2. This interdependence of the two control regions emphasized the importance of ETS2 as a transcriptional regulator of *CGA* expression. The experiments also confirmed the broad role that ETS2 apparently plays in the up-regulation of signature genes of trophoblast from a wide range of species (Ghosh, Sachdev et al. 2005).

Yet another transcription factor has been implicated in controlling *CGA* expression in choriocarcinoma cells (Roberson, Meermann et al. 2001). *DLX3*, as demonstrated by electrophoretic mobility shift assays (EMSA), associated with a sequence (-114 to -107) that overlaps the OCT4 binding region within the junctional regulatory region (JRE) (see below). Over-expression of *DLX3* modestly up-regulated the *CGA* promoter (~ 2.5 fold). Site-directed mutagenesis of the octamer site reduced the basal activity of the *CGA* promoter, suggesting the possible importance of this site. Moreover, binding of *DLX3* to the octamer site is necessary for transactivation of the *CGA* promoter.

In 1997, Liu et al. reported an OCT4 binding site (ATGGTAAT) spanning the region -117 to -110 bp upstream of the transcription start site of the human *CGA* gene.

They also showed that OCT4 strongly silenced *CGA* promoter activity and reduced the production of *CGA*-encoded protein by ~ 90 % (Liu, Leaman et al. 1997). However, mutation of the OCT4-binding site did not interfere with the ability of OCT4 to silence promoter activity, suggesting that OCT4 might not be required to associate with the DNA to exert its effects on transcription. Liu et al. (Liu and Roberts 1996; Liu, Leaman et al. 1997) proposed that the down regulation of OCT4 in the emerging trophoblast was likely to be a prerequisite for the eventual expression of both CG subunit genes and the production of hormone.

Although not pursued here, OCT4 effectively silences reporter gene expression driven by the *CGB* as well as the *CGA* promoter (Liu and Roberts 1996; Liu, Leaman et al. 1997). In addition, it effectively down-regulates the promoter of *IFNT* genes, whose protein products are responsible for rescuing the corpus luteum of pregnancy in ruminant species (Ezashi, Ghosh et al. 2001), suggesting that OCT4 might have a broad function in controlling the transcriptional activity of several signature genes of the trophoblast prior to the establishment of this lineage. OCT4 contains a bipartite DNA binding domain (a 75 amino acid N terminal POU specific and a 60 amino acid C terminal POU homeodomain) (Pan, Chang et al. 2002) (Fig. in chapter I). In the case of *bIFNT*, silencing was accomplished by OCT4 associating with ETS2, which probably accounted for the failure of the latter to transactivate the *IFNT* promoter (Ezashi and Roberts 2004). Based on these data, Ezashi et al. (Ezashi, Ghosh et al. 2001) proposed that OCT4 silences ETS2 mediated transactivation of *IFNT* promoter by a quenching mechanism (Ezashi, Ghosh et al. 2001).

Here I describe experiments that provide a better understanding of how OCT4 silences *hCGA* through its interaction with ETS2 and how ETS2 and DLX3 act cooperatively in controlling expression driven by the *CGA* promoter.

Materials and Methods:

Reporter gene constructs and expression plasmids:

Human *CGA* promoter constructs driving the luciferase (luc) reporter gene (-255luc), containing the gene control region -255 to +48, were subcloned into pGL2 basic vector (Promega, Madison, WI).

The expression plasmids for mouse *OCT4*, as well as its derivatives *pCMV-OCT4*, *pCMV-4N-POU4*, *pCMV-4N*, *pCMV-POU4*, and *pCMV-POU4-4C* were provided by H. Schöler, currently based at Max Planck Institute for Molecular Biomedicine, Münster, Germany (Ezashi, Ghosh et al. 2001). The expression plasmid for ETS2 has been described previously (Ezashi, Ealy et al. 1998). A construct with the OCT4-binding site mutated was prepared in the manner described previously (Liu, Leaman et al. 1997). The *DLX3* expression plasmid *DLX3/pCI-neo* was obtained as a gift from Dr. Maria Morasso (National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD) and has been described elsewhere (Morasso, Grinberg et al. 1999; Roberson, Meermann et al. 2001).

Cell culture and transfections:

JAr choriocarcinoma cells (HTB-144; American Type Culture Collection, Manassas, VA) were maintained in RPMI-1640 (Invitrogen, Carlsbad, CA) medium supplemented with 10% fetal bovine serum (Harlan, Indianapolis, IN). The cells were transfected by using Lipofectamine - Plus (Invitrogen) reagents as per manufacturer's instructions. JAr cells were plated in 6-well plates (TPP/Midsci, St. Louis, MO) (1×10^5 cells/well), incubated overnight and transfected with 0.5 ug of reporter gene construct and 1.5 ug of expression vector DNA per well in presence of 25 ng of pRSVLTR- β gal. Total amount of transfected DNA was kept constant. After 44 h of incubation at 37 °C under 5 % CO₂, the cells were washed with PBS (Gibco, Invitrogen, Carlsbad, CA) and lysed with Tropix, Galactolight-plus lysis buffer (Applied Biosystems, Foster City, CA). *Luc* activity was measured by luciferase reagent (Promega, Madison, WI) with a 20/20ⁿ luminometer (Turner Biosystems, Sunnyvale, CA). Beta-galactosidase activity was measured by using Tropix Galactolight™ Substrate (Applied Biosystems). Extracts were heated at 48°C for 1h to inactivate endogenous β -galactosidase. The enzymatic activities of each promoter *luc* reporter construct were normalized to the control β -galactosidase activity. JAr cell-lines stably transfected with pcDNA3-Oct4 expression vector (clones S1 and S4) and with pcDNA3 empty vector (clones C1 and C2) have been described previously by Liu et al (Liu and Roberts 1996; Liu, Leaman et al. 1997). These cell lines were maintained in 90 % DMEM supplemented with 10 % fetal bovine serum.

Western blot analysis:

Whole cell lysates were prepared from normal JAr cells, as well as from the stably transfected JAr lines S1, S4, C1 and C2 cells, by using RadioImmunoPrecipitation Assay (RIPA) buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 0.1 % SDS (w/v), 1 % Triton X-100 (w/v) and 1X protease inhibitor cocktail (Sigma, St. Louis, MO). Lysates were centrifuged (4000 x g) to remove particulate matter. Cleared cell lysates were analyzed by 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Pre-stained dual label protein ladders (Biorad) were used as mol wt markers. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA). Affinity-purified sheep anti-DLX3, rabbit anti-OCT4 or rabbit anti-ETS2 antibodies (raised in our laboratory) were diluted 1:1000 before use. Rabbit anti- β -actin antibody (Cell Signaling Technologies, Danvers, MA) was diluted 1:1000. Secondary antibodies used were HRP-linked anti-sheep IgG or HRP-linked anti-rabbit IgG (Cell Signaling Technologies, Danvers, MA) diluted 1:5000. All antibody dilutions were made in 5 % non-fat dry milk (NFDM) (w/v). Membranes were developed with Photo-type Horseradish Peroxidase western blot detection system (Cell Signaling Technologies). Images were acquired with the Fuji LAS 3000 Imaging system (Fujifilm Medical Systems, Stamford, CT). Some of the blots were stripped with Restore-Plus Western Blot stripping buffer (Thermo Scientific, Rockford, IL), blocked with 5 % non-fat dry milk (w/v) and re-probed with a different antibody. The Manufacturer's protocol provided by Thermo Scientific was followed.

Chromatin immunoprecipitation analysis:

ChIP analysis from nuclear extracts of JAr choriocarcinoma cells was performed as described earlier (Das, Ezashi et al. 2008). Briefly, sheared chromatin was obtained from stably transfected JAr S4 and C2 cells and exposed to 200 μ l slurry of Protein G-agarose beads (Santa Cruz Biotechnology). One-fifth of the total volume was preserved at -80°C as “total input” control. The remaining chromatin was subdivided into a number of treatment groups, e.g. untreated (“no antibody” control), exposed to a specific antibody (3 μ g of rabbit anti-OCT4 immunoglobulin or rabbit anti-ETS2 immunoglobulin, or exposed to purified IgG from a non-immunized rabbit (Active motif, Carlsbad, CA) respectively. The immune complexes were collected on Protein G-agarose beads (Santa Cruz Biotechnology), eluted by using elution buffer, subjected to vortexing for 20 min and prepared for PCR analysis. The primers used are shown in Table 1 (Chapter III) and were designed to amplify a region of the *CGA* proximal promoter (-96 to +49) containing the ETS2 binding site. PCR conditions were as follows: 95°C for 2 min for one cycle, 25 cycles of 95°C for 20 sec, 50°C for 20 sec, 72°C for 50 sec, followed by 72°C for 5 min. PCR products were visualized by ethidium bromide staining after electrophoresis on 1.5% agarose gels. A second set of ChIP assays were performed by using sheared chromatin obtained from normal JAr cells transiently transfected at a range of OCT4 expression vector concentrations. Affinity-purified rabbit anti-ETS2 antibody was used to detect any endogenous ETS2-DNA complex. PCR analysis was performed as above. To study the interaction between DLX3 and OCT4, a similar protocol was followed. However, the complex was immunoprecipitated with ChIP grade goat polyclonal DLX3

antibody (N-17-X) (Santa Cruz Biotechnology) and a different set of primers were used (Table I, Chapter III).

Co-immunoprecipitation analysis:

JAr choriocarcinoma cells grown in 60 mm diameter dishes were transiently transfected with 3 µg of *pCGN-ETS2* and 3 µg of *DLX3/pCI-neo* expression plasmid DNAs by using Lipofectamine/Plus reagents. Extracts were prepared from each set of reactions by using RIPA lysis buffer. Cell lysates were cleared by centrifugation at 4000 x g. Immunoprecipitation reactions were started with fresh cell extracts (~ 600 µl) containing 1 mg protein. After treating with Protein G agarose beads, the lysates were incubated overnight with either 5 µg of affinity-purified anti-ETS2 antibody (sc-351) or purified nonspecific IgG (Santa Cruz Biotechnology). Next day, immunoglobulin complexes were adsorbed onto 50 µl of swollen, pre-washed Protein G agarose beads for 6 h. The bound immune complexes were eluted in non-reducing sample buffer (Laemmli buffer without 2-mercaptoethanol) at 80 °C for 15 min. Samples were analyzed in 12% SDS - PAGE gels. The immune complexes formed with ETS2 antibody were detected by western blot analysis on 25 µg of protein, with affinity-purified sheep DLX3 immunoglobulin (diluted 1:1000 in 5% non-fat dry milk) (w/v) as the detecting antibody. The bands of DLX3 were visualized by chemiluminescence with Lumiglo and Peroxide reagents purchased from Cell Signaling Technologies. Coimmunoprecipitation studies were also studied in reverse where the complex

was immunoprecipitated with affinity-purified DLX3 antibody and western analysis was performed with affinity-purified ETS2 antibody.

Immunofluorescence studies:

JAr cells were grown on cover-slips placed in six-well plates. After fixation with paraformaldehyde and permeabilization with 1% Triton (w/v), the cells were incubated overnight with rabbit anti-ETS2 (1:200) and sheep anti-DLX3 antibody (1:40). Following secondary incubation with respective Alexa Fluor labeled antibodies, the nuclei were stained with DAPI. The slides were mounted on coverslips and studied under Olympus microscope.

Statistical analyses:

Transient transfection assays were repeated either two or three times, each with triplicate samples. The data from these independent transfections were analyzed either by pair-wise comparison with a student *t* test or by one-way analysis of variance followed by Tukey's multiple comparison test (GraphPad Prism version 4, GraphPad Software Inc, San Diego, CA). *P* values of < 0.05 were considered significant.

Results:

OCT4 effectively silences CGA promoter activity:

Fig. 2 depicts the organization of the *hCGA* promoter and its ETS2 and OCT4 binding sites. JAr cells were transiently co-transfected with the *CGA-luc* wild type

reporter construct and the ETS2- and OCT4-expressing constructs, either alone or in combination. ETS2 modestly, but significantly ($P<0.05$) up-regulated the *CGA* promoter 3-4 fold (Fig. 2A). OCT4, on the other hand, repressed the basal as well as ETS2-mediated transactivation of the *CGA* promoter by $\sim 90\%$ ($P<0.0001$), re-emphasizing the potential role of OCT4 in silencing *CGA* (Fig. 2A). To determine the potential importance of the OCT4-binding element (-117 ATGGTAAT - 110) in the upstream junctional regulatory region in controlling expression from the *CGA* promoter, it was mutated (-117 ATGGTACG - 110) at two sites. Basal expression from this construct was reduced approximately $\sim 90\%$ compared to the wild-type promoter with the intact octamer site (Fig. 2B). Although ETS2 had only a modest ability (1.5 - 2-fold; $P<0.01$) to up-regulate the activity of the *mut-OCT(-255luc)* promoter, OCT4 could still effectively repress ($P<0.0001$) basal expression from the mutated promoter (Fig. 2B). This result suggests that OCT4 does not need to bind to its octamer sequence to repress *CGA-luc* reporter activity. The data also suggest that mutation of the octamer sequence (Fig. 2C) might be interfering with the ability of a second transcription factor to transactivate the *CGA* promoter. In addition, the experiments imply that OCT4 interferes with ETS2-mediated transactivation without binding to the DNA itself, at least at its only known binding site.

Since transcription factors exert their action either through binding to DNA or through their interactions with other proteins, they act in a dose-dependent manner that is amenable to thermodynamic and kinetic analysis. To determine the dose dependency of OCT4 repression of ETS2-mediated *CGA* promoter transactivation, the ability of OCT4 expression to repress the promoter-reporter activity was examined over an extended

range of expression plasmid concentrations, while keeping the concentrations of -255luc and ETS2 expression plasmids constant (Fig. 3). The silencing of reporter gene expression was highly sensitive to OCT4 inhibition, with effects detectable at concentrations as low as 0.5 ng/ml plasmid and half-maximal inhibition noted at 2.5 ng/ml. This concentration of plasmid vector was chosen in all subsequent experiments to allow comparisons to be made between different domains of OCT4 in their relative effectiveness as inhibitors and to study potentially competing effects of other transcription factors.

Domain specificity of OCT4 required for silencing of hCG α promoter activity.

OCT4 protein is comprised of an N-terminal (transactivation) domain, a C-terminal domain and a POU (DNA binding) domain. To determine the role of each domain in silencing *CGA* promoter activity, expression plasmids lacking various domains, but driven by the same promoter, were co-transfected with the ETS2 expression vector and the -255luc reporter. All the OCT4 vectors, including the full length one, were used at a concentration of 2.5 ng/ml. Consistent with the results shown in Fig. 2A, ectopic expression of ETS2 alone transactivated the promoter approximately 4-fold (Fig. 4A). Whereas the full length OCT4 construct again inhibited ETS2-activated reporter expression by about 50 %, neither the POU, N-terminal, nor the POU-carboxyl terminal domains together had any inhibitory effect. The only sub-domain construct observed to cause inhibition was the one that expressed both the N-terminal and POU domains together. At 2.5 ng/ml (5 ng/dish) the N-terminal-POU construct was as an effective a silencer as the one expressing full length OCT4. These data suggest that presence of both

the N-terminal and POU domains are required for effective silencing of the *CGA* promoter.

To test this conclusion further, inhibition by the N-POU construct was tested over a series of increasing transfection concentrations. An inhibition curve comparable to that of full length OCT4 was observed (Fig. 4B). Expression of the N-terminal-POU domain also silenced ETS2-driven reporter gene expression from the *CGA* promoter with the mutated octamer site, again stressing that this binding site is not required for OCT4 inhibition of *CGA* but is necessary for ETS2-mediated transactivation.

Regulation of CGA promoter activity by DLX3:

The next objective was to identify the likely candidate transcription factor that interacted with the *CGA* promoter close to the octamer binding site and that was also implicated in enhancing the ability of ETS2 to drive transactivation of reporter gene expression from the *CGA* promoter. DLX3 was immediately suspected because it was known to bind within the upstream JRE region of the *CGA* promoter (Roberson, Meermann et al. 2001), which also includes the OCT4-binding site. Moreover DLX3 partners with ETS2 to up-regulate *IFNT* expression (Ezashi, Das et al. 2008).

To test whether DLX3 might be the candidate factor, JAr cells were transiently transfected with *DLX3* and *ETS2* expression vectors, either alone or in combination. As observed previously, ETS2 up-regulated the promoter 3-4 fold, while DLX3 alone only increased reporter expression 2-2.5 fold. However, co-expression of ETS2 and DLX3 synergistically up-regulated the *CGA* promoter activity 20-22 fold (Fig. 5), suggesting the importance of both transcription factors acting together in regulating the promoter.

To determine the dose dependency of DLX3 and ETS2-mediated transactivation of *CGA* promoter activity, the ability of DLX3 expression to transactivate the promoter-reporter activity was examined over an extended range of expression plasmid concentrations, while keeping, the concentrations of *-255luc* and ETS2 expression plasmids constant. With increasing concentrations of DLX3 expression vector, up-regulation of *CGA* promoter activity increased from ~ 20 fold to 1000 fold (Fig. 6), suggesting a sensitive and powerful mechanism for regulating *CGA* involving DLX3 and ETS2.

To determine whether this combinatorial, synergistic effect of DLX3 and ETS2 is altered upon mutation of either DLX3 (Fig. 7A) or ETS2 (Fig. 7B) binding sites, separate transient transfection assays were performed with either the DLX3 binding site mutated or one of the ETS2 binding sites mutated. Compared to the wild-type *CGA* promoter, the synergistic up-regulation was completely abolished with either of the mutated *CGA* promoters, suggesting that both transcription factors need to bind to their respective binding sequences to transactivate the *CGA* promoter. Moreover, a gradual reduction in synergism was observed when transient transfection assays were performed with progressively deleted *CGA* promoter constructs (Fig. 8A). The longest promoter construct, which contained both the intact DLX3 and ETS2 binding sites, was able to up-regulate reporter gene activity ~ 22 – 24 fold upon co-expression of DLX3 and ETS2. However, a shorter promoter lacking the DLX3 binding site provided much reduced (35 – 40 %) reporter gene expression. Transient transfection with the shortest promoter, lacking both the DLX3 and ETS2 binding sites gave very low activity and no evidence for synergistic cooperation of the two transactivators (Fig. 8B). These observations support

the previous data and suggest the importance of both an intact DLX3 and ETS2 binding site for full transactivation of the *CGA* promoter.

Coimmunoprecipitation of DLX3 and ETS2 from JAr cell extracts:

So far, the experimental data imply that both DLX3 and ETS2 can associate on the *CGA* promoter in JAr cells. To determine whether these two proteins exist together as a complex, co-immunoprecipitation analysis was performed. Cell lysates were prepared, from cells transiently transfected with ETS2 and DLX3, either alone or in combination. Whole cell lysates (1 mg protein) were pre-cleared with 100 μ l of protein G agarose beads (Santa Cruz Biotechnologies) and incubated with either affinity-purified ETS2 antibody or a non-specific IgG. Protein complexes were eluted and analyzed on 12 % SDS - PAGE gels. A sample of whole cell lysate (25 μ g protein) was analyzed as a positive control in the western blotting. Protein detection on the blots was performed with affinity-purified DLX3 antibody. DLX3 was detected in the immunoprecipitated ETS2, with the band intensity increasing in the lysates that contained over-expressed ETS2 and DLX3 (Fig. 9). Coimmunoprecipitation reaction was also performed in the reverse experiment where the complex was immunoprecipitated with affinity-purified DLX3 antibody and western analysis performed with ETS2 antibody. The band intensity of ETS2 was again stronger in the lysates that over-expressed ETS2 and DLX3, compared to either the whole cell lysate or the immunocomplexes collected from non transfected cells. This result demonstrates that DLX3 and ETS2 form a complex stable enough to be

immunoprecipitated from JAr cells. Moreover, the two proteins co-localize in the JAr cell nucleus (Fig. 10).

Expression of ETS2, DLX3, and OCT4 in JAr S and C cell-lines:

Whole cell lysates were prepared from the non-transfected JAr, S1, S4, C1 and C2 cell-lines described in more detail in Chapter 3. The S1 and S4 cells stably express OCT4. As before the cell lysates were subjected to Western blotting analysis. When compared to the band of β -actin used as a loading control, it was clear that all of the cell-lines expressed similar amounts of ETS2 and DLX3 (Fig. 11A), while OCT4 was detectable only in S1 and S4 cells, but not in C1 and C2 (Fig. 11B). This experiment suggests that OCT4 expression does not alter the expression of DLX3 and ETS2, only their downstream target genes.

OCT4 silences CGA promoter activity by interfering with ETS2 binding:

We performed chromatin immunoprecipitation analysis using chromatin obtained from S and C cells to determine the binding efficiency of ETS2 to the *CGA* promoter in presence or absence of OCT4. ETS2 failed to form a complex with the *CGA* promoter in presence of OCT4 (S cell chromatin). However, a strong ETS2-DNA complex was observed in absence of OCT4 (C cell chromatin) (Fig. 12A). It would appear that OCT4 physically interacts with ETS2 while the latter is not associated with the *CGA* promoter sequence, thereby interfering indirectly with normal binding to the ETS2-binding sequence. Such data are more consistent with a squelching as opposed to a quenching

mechanism of OCT4-mediated repression. A schematic model representing OCT4-mediated suppression of ETS2 is shown in Fig. 13.

In addition, ChIP analyses were performed on chromatin from control JAr cells that had been transiently transfected with a range of concentrations of OCT4 expression plasmids. ETS2 formed a stable complex with the *CGA* promoter in the absence of OCT4, but failed to form such a complex at OCT4 plasmid concentrations as low as 10 ng/ml (Fig. 12B). This result supports the previous data that ETS2-mediated transactivation of the *CGA* promoter is extremely sensitive to even low expression of OCT4.

OCT4 interferes with DLX3 – mediated transactivation of the CGA promoter:

The previous experiments demonstrated that OCT4 silences both basal as well as ETS2 – mediated transactivation of the *CGA* promoter activity. The next objective was to determine if DLX3 – mediated activation of the *CGA* promoter can also be repressed by OCT4. Transient transfection assays were performed to determine the effect of a wide range of OCT4 expression plasmid concentrations on DLX3–mediated up-regulation of the *CGA* promoter. As observed with ETS2, the DLX3-mediated transactivation was silenced in a dose-dependent manner by increasing OCT4-expression plasmid concentrations (Fig. 14). However, whereas a concentration of OCT4 plasmid, as low as 2.5 ng / ml was sufficient to reduce ETS2-driven transactivation by ~ 50 %, a significantly higher concentration of OCT4 expression plasmid (~ 1 µg) was required to prevent DLX3 – mediated transactivation of the *CGA* promoter activity by half. As DLX3 shares a binding site on the *CGA* promoter that overlaps that occupied by OCT4 (Fig. 13B), it seemed possible that OCT4 interfered with DLX3 transactivation by

competing for the common binding site. The potency of this inhibition would presumably reflect the binding affinities of the two factors for this DNA sequence.

OCT4 interferes with binding of DLX3 to the CGA promoter:

To determine whether DLX3 can bind to the *CGA* promoter in the presence of OCT4, ChIP analysis was performed with chromatin from S4 and C2 cells. In the absence of OCT4 (C2 chromatin), a strong DLX3 – DNA complex was observed. On the other hand, the amount of DLX3 complexed with DNA was reduced in chromatin isolated from C cells, which stably expressed OCT4 (Fig. 15). This finding provides additional support for the hypothesis that OCT4 interferes with DLX3 transactivation, not by a squelching or quenching mechanism but by competing for the DLX3 binding site on the promoter.

Discussion:

Biologically active CG consists of two subunits, one encoded by *CGA* and the other one by *CGB* genes. The *CGA* subunit, which is common to LH and TH as well as CG, is encoded by a single locus (Fiddes and Goodman 1979; Boothby, Ruddon et al. 1981) and has been regionally mapped to human chromosome 6q12 – q21 (Naylor, Chin et al. 1983). The *CGB* subunit is encoded by at least 6 genes or pseudo-genes, which are located at chromosome 19q13.3 in a common genome cluster that also includes *LHB* (Policastro, Daniels-McQueen et al. 1986; Rull, Hallast et al. 2008). In placenta, *CGB5* is predominantly expressed, with smaller amounts of *CGB3* transcripts as well (Policastro, Daniels-McQueen et al. 1986). Accordingly, the expression of *CGA*, *CGB* and of the

second gene *CGB3*, which encodes an identical polypeptide to *CGB5*, would be expected to exhibit some degree of coordination to provide an adequate balance of the two subunit proteins. As expected, therefore, some of the transcriptional control mechanisms operating over *CGA* and *CGB* appear to be similar. For example, ETS2 is a major transcriptional activator of several “signature” genes of trophoblast (Hemberger and Cross 2001) including *CGA* and *CGB* subunit genes in humans (Johnson and Jameson 2000; Ghosh, Ezashi et al. 2003; Ghosh, Sachdev et al. 2005) as well as *IFNT* in ruminants (Ezashi et al, 1998). In addition, cAMP up-regulates both subunit genes in a dose-dependent manner (Andersen, Kennedy et al. 1990; Steger, Buscher et al. 1993; Ghosh, Sachdev et al. 2005), although the mechanisms of regulation may be quite different. OCT4 is also a negative regulator of *CGA* and *CGB* subunit genes (Liu and Roberts 1996; Liu, Leaman et al. 1997). Despite these similarities in control, the sequences of the *CGA* and *CGB* gene control regions (Fig. 8 and Fig. 9 in Chapter I) show little or no resemblance when they are aligned, although a few common motifs such as ETS2 binding sites are recognizable. Such observations suggest that both genes have evolved separately to provide trophoblast expression and that there are likely to be circumstances where expression of one exceeds that of the other. Such differences are, in fact, observed. As noted in Chapter III, transcript concentrations of *CGA* mRNA exceed *CGB* by over ~ 10 fold in JAr cells and in pregnant women. Although intact CG appears first in the serum, with time there are increases in amounts of free subunits, with that of *CGA* greatly exceeding both *CGB* and intact hormone after the first trimester (Ozturk, Bellet et al. 1987). These results emphasize that different controls, most likely transcriptional, must be operating on the two genes. The data also suggest that the free

subunits may have roles that are distinct from that of the heterodimer. For example, CGA promotes the release of prolactin from endometrial decidual cells and hence may have indirect effects on mammary development and lactation (Han, Lei et al. 1999). Accordingly, each subunit gene is likely to show differences as well as similarities in transcriptional control. In this chapter emphasis has been on the alpha subunit gene, *CGA*, and particularly the role of OCT4 as a repressor and DLX3 as an activator.

The POU domain transcription factor OCT4 is considered as the master regulator of stem cell self-renewal and pluripotency and is present in the inner cell mass of mammalian embryos, embryonic germ cells and cultured embryonic stem cells (Hansis, Grifo et al. 2000). Maintenance of OCT4 at a critical concentration is probably required for self-renewal of pluripotent cells. In mouse embryos, over-expression of OCT4 causes differentiation towards endoderm lineage while its suppression triggers differentiation towards trophectoderm (Nichols, Zevnik et al. 1998). Similarly, knockdown of OCT4 in murine and human embryonic stem cells causes the cells to convert to trophoblast-like cells (Loh, Wu et al. 2006; Babaie, Herwig et al. 2007). However, in early embryos of all mammals that have been examined, including mouse, human, pig and cow, OCT4 is not confined to the inner cell mass, but continues to be expressed temporarily in trophectoderm, where it can presumably continue to influence gene expression (Scholer, Dressler et al. 1990; Hansis, Grifo et al. 2000; Brevini, Tosetti et al. 2007). Its expression in trophectoderm diminishes as development proceeds and is usually absent after the embryo hatches from the zona pellucida (Palmieri, Peter et al. 1994; Hansis, Grifo et al. 2000). There has been much discussion as to whether the up-regulation of the caudal transcription factor CDX2 in outer cells of the embryo and the

accompanying down-regulation of OCT4 in these cells are linked and key to driving emergence of trophoctoderm (Strumpf, Mao et al. 2005; Kunath, Saba-El-Leil et al. 2007). Whether or not this hypothesis is correct or not, the onset of expression of various trophoblast-specific genes including *CG* and *IFNT* that are potentially silenced by OCT4 (Liu and Roberts 1996; Liu, Leaman et al. 1997; Ezashi, Ghosh et al. 2001) appears to coincide with the down-regulation of this transcription factor. Hence, it might be expected that *CGA* and *CGB* subunit gene transcription will rise as OCT4 expression falls. According to this model, it seems possible that OCT4 has a broad role in preventing the expression of many genes whose early up-regulation in the inner cell mass might interfere with the capacity of these cells to remain pluripotent. Such a model is somewhat consistent with the roles played by OCT4, NANOG and SOX2 in embryonic stem cells in suppressing the expression of genes that are essential for emergence of the three main germ layers and more differentiated lineages (Chen, Vega et al. 2008; Pei 2009).

Dose-dependent repression of *CGA* promoter activity (Fig. 3) demonstrates that a very low concentration of OCT4 is sufficient for silencing activity, which is dependent on the presence of the POU and N-terminal domains of the protein, with no requirement for the amino terminal domain (Fig. 4). This result is consistent with previous studies on *IFNT* gene regulation where the same domains were necessary for silencing (Ezashi, Ghosh et al. 2001). As OCT4 effectively represses endogenous *CGA* and *CGB* subunit gene expression in JAr cells and reduces both transcript and protein concentrations by more than 80 % (Liu and Roberts 1996; Liu, Leaman et al. 1997), it seemed logical to expect the mechanism of silencing to be similar, but this was reported not to be the case (Liu and Roberts 1996; Liu, Leaman et al. 1997). Instead, OCT4 may repress the two

genes by different means. In the case of *CGA*, mutation of the binding site for OCT4 did reduce both baseline and ETS2-mediated transactivation of the gene (Fig. 2), but OCT4 silencing effects remained unchanged in the sense that the dose-response effects did not change (Fig. 3). In contrast, Liu & Roberts et al (Liu and Roberts 1996) reported that mutation of the octamer site in the *CGB* gene abolished OCT4 silencing. However, when these experiments were repeated (R. Gupta, unpublished data) with a much more sensitive reporter gene (*LUC* versus *CAT*) for assessing promoter activity, in contrast to the earlier work (Liu and Roberts 1996), the same mutation was found not to affect the silencing ability of ectopically expressed OCT4. The explanation for these contrasting results is unclear, but in view of these more recent experiments it remains possible that OCT4 silencing of the *CGA* and *CGB* genes operates through a similar mechanism after all. Instead of being dependent on the octamer binding site, it would appear that OCT4 silencing operates through a mechanism that involves interference with ETS2-mediated transactivation (Fig. 12). The POU domain of OCT4 has been previously shown to form a stable complex with the transactivation domain (a site located between the “POINTED” and DNA binding domain) of ETS2 (Ezashi, Ghosh et al. 2001). From the present study, it is clear (as demonstrated by ChIP analysis) that OCT4 over-expression interferes with the ability of ETS2 to bind to the *CGA* promoter (Fig. 12), thereby providing the likely explanation for the OCT4 silencing effect. The ChIP analysis also confirms the great sensitivity of ETS2 binding to low concentrations of OCT4. As OCT4 would appear to sequester free ETS2 rather than ETS2 bound to the promoter, the mechanism of silencing is by “squenching” as opposed to quenching. In the latter, the repressor and transactivator remain associated with the DNA, with inhibition occurring through interference with the

transactivation process (Levine et al, 1989). Squelching appears to constitute a relatively common mechanism for repressing gene expression. For example, a dominant negative c-JUN can squelch AP1 – mediated and other signaling responses in basal cell carcinoma cells (Thompson, Gupta et al. 2002). Interaction between different steroid receptors and coactivators can also lead to inhibitory outcomes via a squelching mechanism (Zhang and Teng 2001).

It seems likely that OCT4 repression of *IFNT* genes (Ezashi, Ghosh et al. 2001) also involves squelching of ETS2. Presumably any gene that depends strongly upon ETS2 for expression would also be silenced when OCT4 is highly expressed. The OCT4 transcripts and possibly the translated gene product are expressed at very high levels compared to other transcription factors, including NANOG and SOX2, in embryonic stem cells (Pan, Chang et al. 2002; Pei 2009) and, by inference, in the inner cell mass of embryos. Conceivably one function is to squelch the activities of a range of genes that might otherwise be up-regulated if its concentration were to fall significantly.

A final question addressed in this chapter was why mutation of the octamer binding site depressed basal as well as ETS2-mediated transactivation of the CGA gene, even though OCT4 silencing was unaffected. The likely explanation appears to be that this site overlaps the binding site for DLX3, a homeobox family transcription factor required for normal placental development in the mouse (Morasso, Grinberg et al. 1999; Roberson, Meermann et al. 2001). It has been demonstrated earlier that transfected DLX3 up-regulated CGA expression in JAr cells by binding to the promoter, in the junctional regulatory region. However DLX3 appeared to be a relatively weak transactivator, increasing expression of a *Luc* reporter only about 2 – 2.5 fold. A similar modest (2.5-

fold) transactivation of the *CGA* was noted here (Fig. 5) when DLX3 was over-expressed. However, the effects of DLX3 were greatly increased when it was co-expressed with ETS2 (Fig. 5). Moreover, as the DLX3 concentration was titrated upwards by raising the concentration of transfected DLX3 expression plasmid, activation of reporter expression could be increased up to 1000-fold or more (Fig. 6). Full cooperative transactivation probably depends upon ETS2 and DLX3, whose binding sites are separated by 26 nucleotides, i.e. roughly 2.6 helical turns, to associate with each other while bound to the promoter and forming a productive complex with the transcriptional machinery. Further experiments are needed to determine how cAMP activation of CREB plays into this association. Changes in concentrations of relevant concentrations of transcription factors such as ETS2 and DLX3 may provide a partial explanation of why *CGA* transcription rises so rapidly early in pregnancy. Interestingly, OCT4, although apparently sharing a binding site with DLX3 on the *CGA* promoter, appeared not to be very effective in reversing the ability of DLX3 to act as a transactivator (Fig. 14). Indeed the modest silencing effects observed could have arisen indirectly through squelching of endogenous ETS2 rather than from competition for the octamer site. Presumably OCT4 competes poorly with DLX3 for this site. Nor has the ability of OCT4 to interact directly with DLX3 been examined, although there is little to suggest that ectopically-expressed OCT4 has an ability to squelch DLX3. Overall, the data support the hypothesis that the silencing effect of OCT4 on the *CGA* promoter is largely, if not exclusively through its association with ETS2 rather than DLX3.

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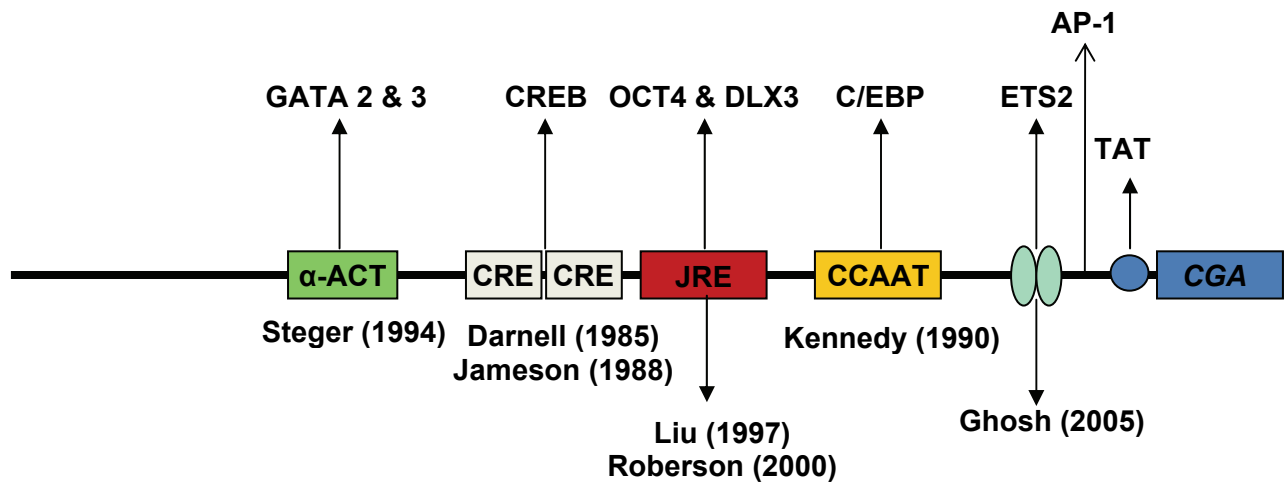
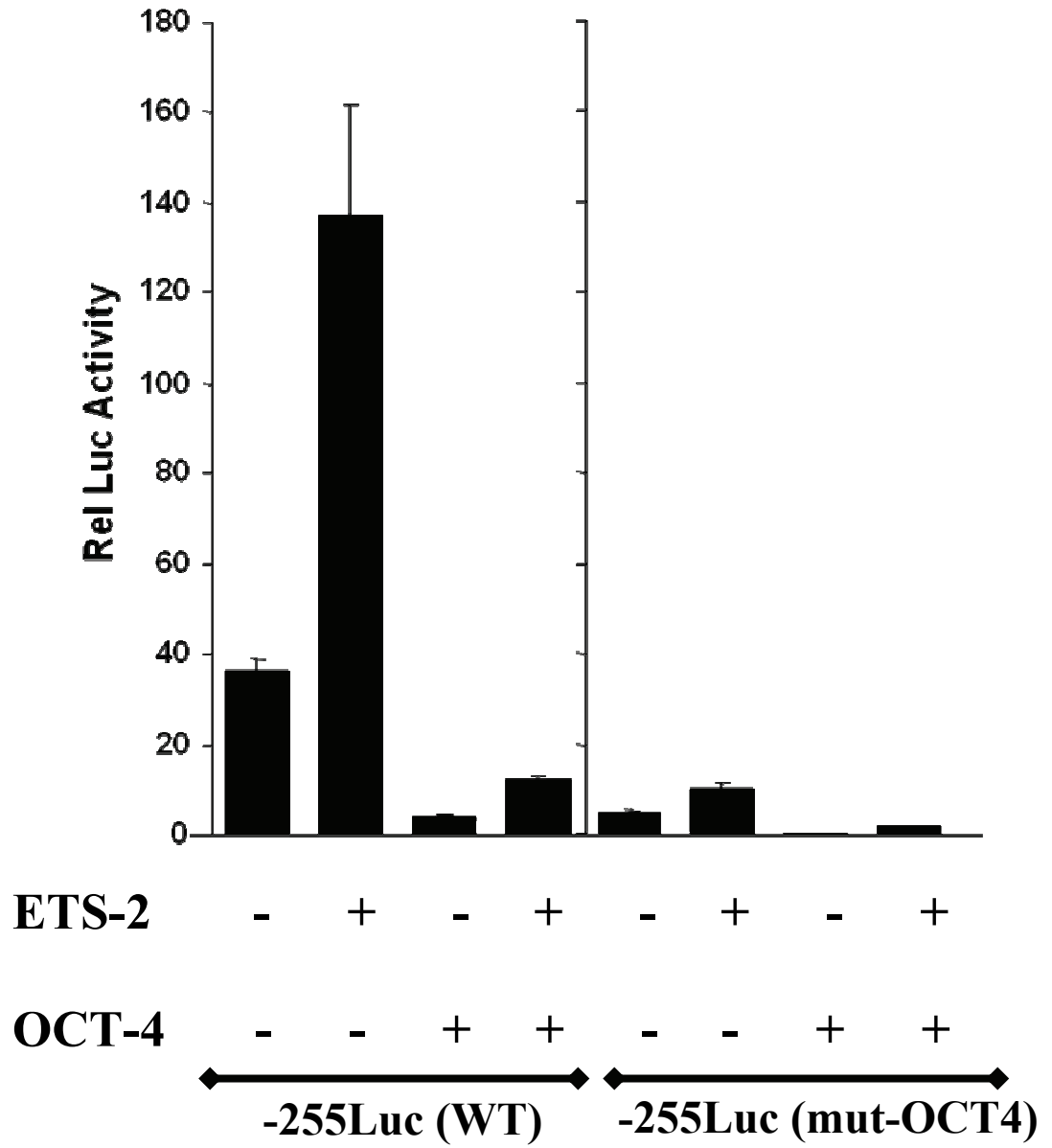


Fig. 1: Schematic representation of the human *CGA* promoter showing important regulatory elements along with their binding sites. Names of the scientists and the years of discovery is also mentioned.



C

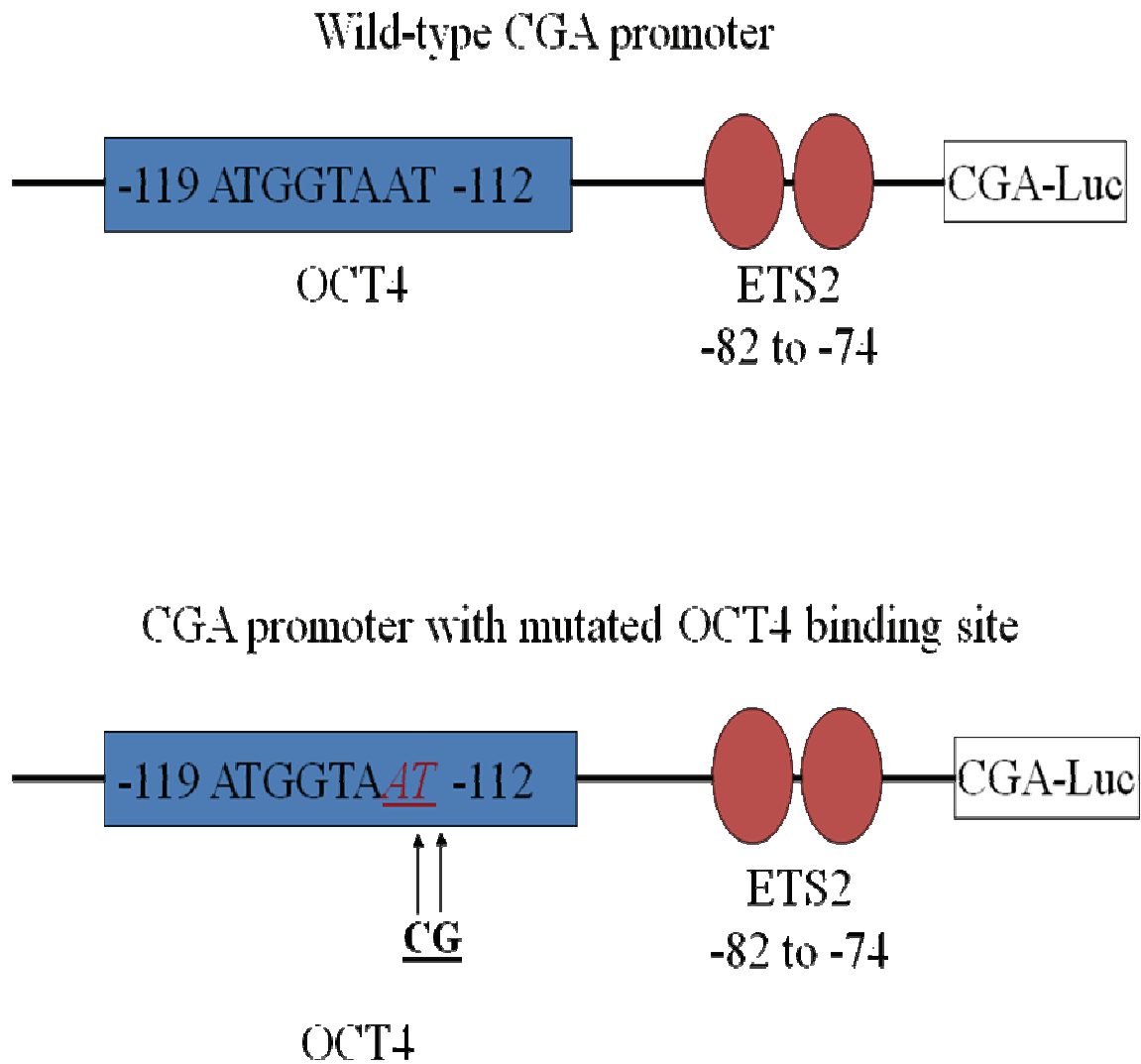


Fig. 2. OCT4 effectively silences human *CGA* promoter activity in JAr choriocarcinoma cells.

(A) The -255 *CGA* promoter-luc reporter construct was transiently cotransfected with ETS2 and OCT4 expression vectors, either separately or in combination into JAr cells. ETS2 modestly up-regulates the promoter by 3-4 fold. On the contrary, OCT4 effectively silences both basal activity as well as ETS2-mediated transactivation by ~ 90 %.

(B) Silencing of human *CGA* promoter by OCT4 is independent of its binding ability to the promoter. The -255luc reporter with OCT4 binding site mutated was compared with the wild-type reporter. Mutation of the OCT4 binding site did not alter the repression of -255luc reporter activity. Both wild-type and mutated basal promoters were significantly ($P < 0.0001$) reduced by OCT4. Data from three independent transfections, each run in triplicate were analyzed by pair-wise comparison using *T* test (Graphpad Prism 4; Graphpad Software Inc, San Diego, CA). The experiments were performed three independent times and their relative luc activit values were log transformed.

(C) Schematic representation of wild-type and OCT4 binding site mutated *CGA* promoter-reporter construct. Two overlapping ETS2 binding sites are located -82 to -74 bp in the proximal part of the promoter. OCT4 binds to an octamer element (ATGGTAAT) spanning -117 to -110 bp upstream of the transcription start site. Site directed mutagenesis has been shown by arrows.

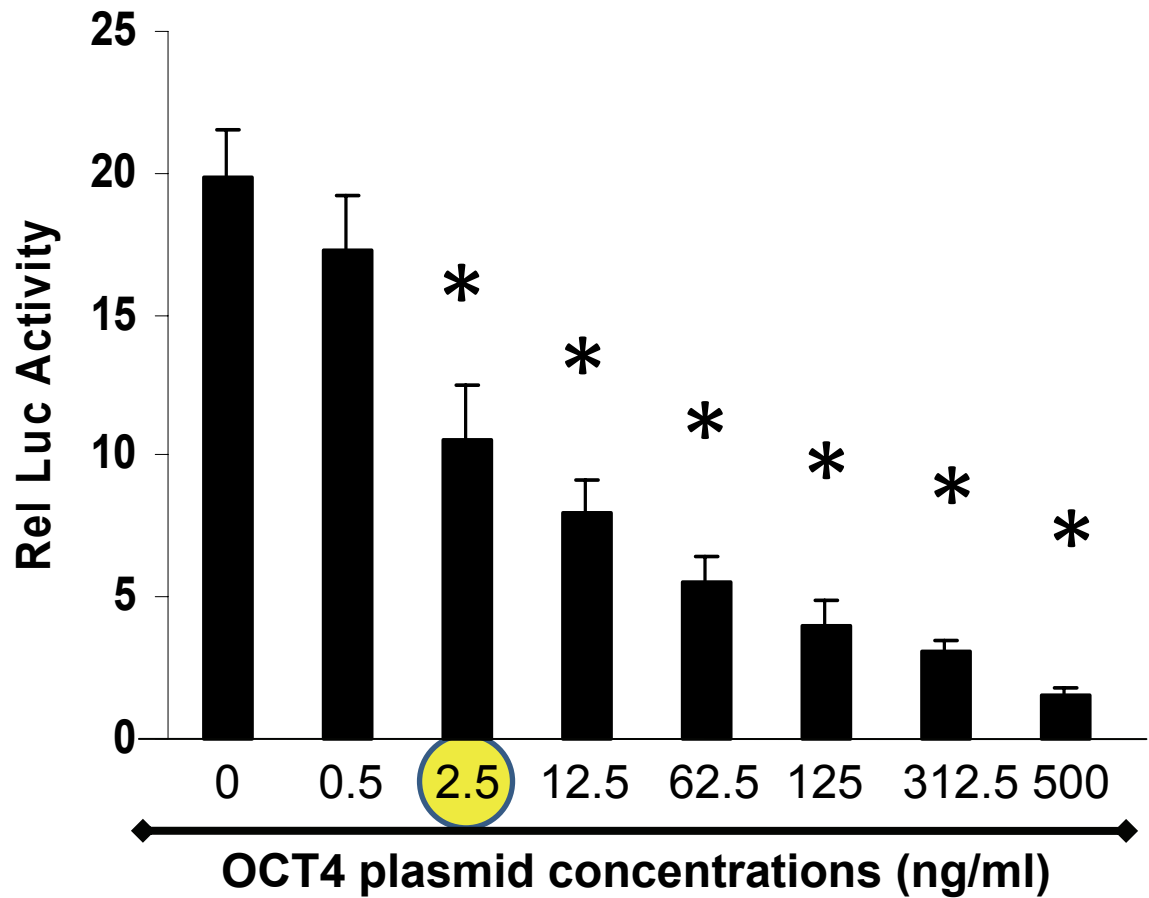
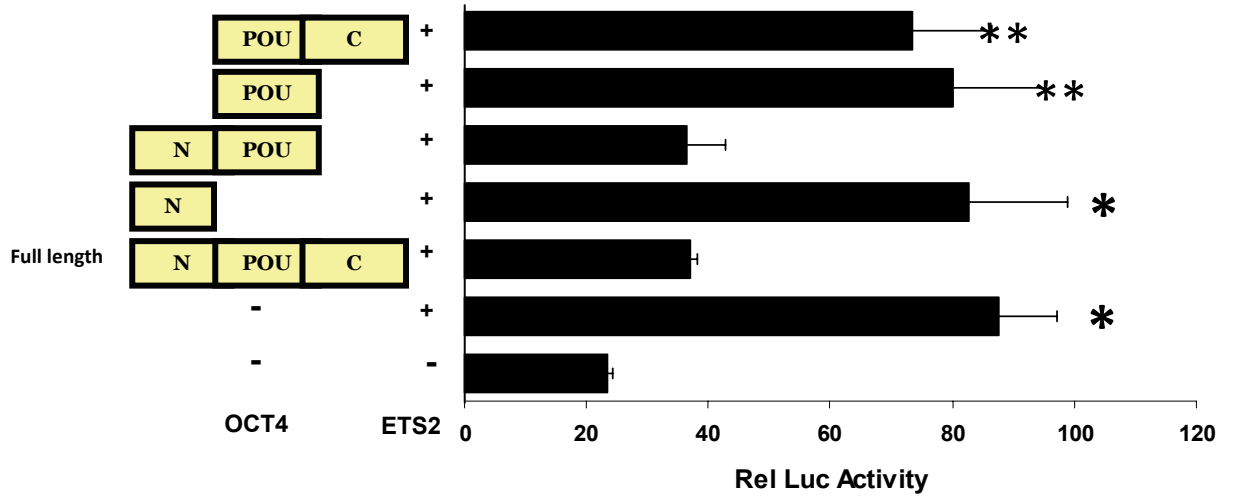


Fig. 3. OCT4 mediated repression of -255 *CGA* promoter is dose-dependent. JAr cells were transfected with -255luc reporter and 0 to 500 ng of OCT4 expression plasmid. pRSVLTR- β gal were cotransfected to normalize the reporter activity. It is clearly evident that as low as 2.5 ng of OCT4 is sufficient for 50% repression of the basal promoter activity. In the subsequent transient transfection assays, this concentration of OCT4 has been used in combination with ETS2 transcription factor. Values marked with (*) differ significantly ($P < 0.001$).

A.



B.

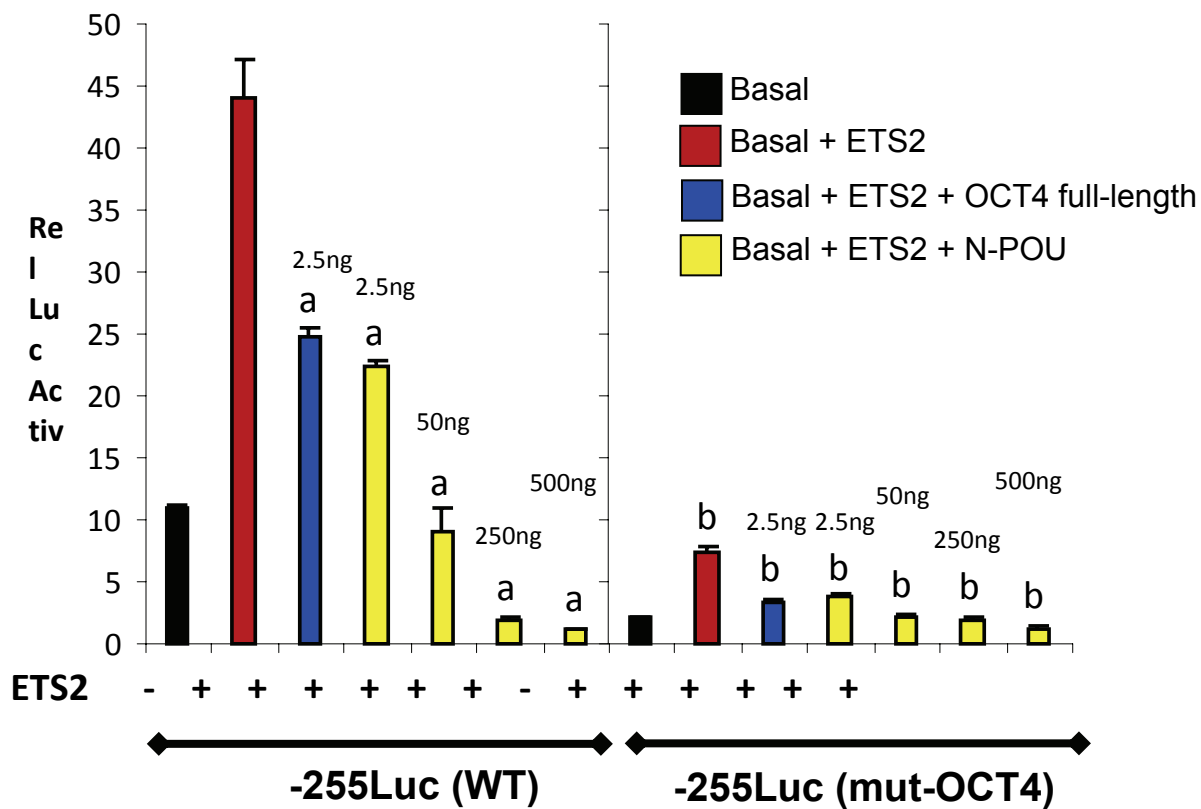


Fig.4. Identification of the domains of OCT4 required for effective silencing of the *CGA* promoter activity.

(A) *CGA-255luc* reporter was transiently transfected either with ETS2 expression vector alone or in combination with various OCT4 deletion constructs. Structures of the various OCT4 deletion constructs have been shown schematically. ETS2 alone modestly up-regulates the basal promoter activity. This transactivation is reduced by almost 50% on co-expression of full-length OCT4. Neither N terminal, nor POU domains alone could repress the ETS2 mediated transactivation. Only presence of both N and POU domains together could effectively repress the transactivation by ~50 %. Values marked with (*) and (* *) differ significantly ($P < 0.01$ and 0.05 respectively).

(B) Dose-dependent repression of *CGA* promoter by OCT 4N-POU deletion construct.

N-POU domains of OCT4 together are sufficient for dose-dependent repression. ETS2 (red bar) modestly up-regulates both basal and mutated promoter. As shown earlier, 2.5 ng of full-length OCT4 represses the ETS2-mediated transactivation of wt and mutated promoter by ~50 % (blue bar). Comparable amount of repression was obtained by 2.5 ng of OCT4 N-POU domains expression vector. Moreover, N-POU domain mediated repression was dose-dependent. The small yellow bars indicate four different concentrations namely 2.5, 50, 125 and 500 ng of N-POU expression vector. The (a*) values are significantly different compared to (a) value ($P < 0.0001$) and similarly, (b*) values are significantly ($P < 0.0001$) different compared to (b) value. The wild-type and the mutated promoters were separately analyzed statistically using one-way ANOVA followed by Tukey's multiple comparison test. Relative luc values of three independent experiments were log transformed.

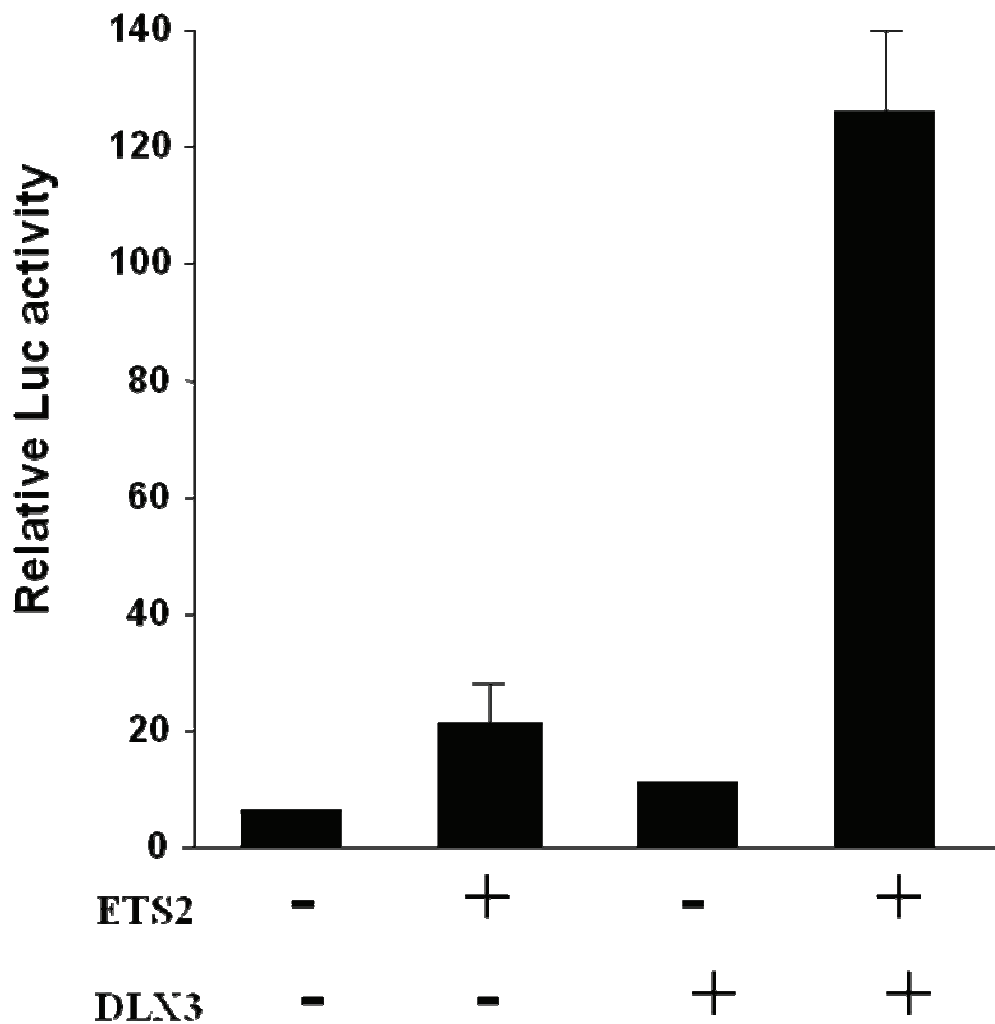


Fig. 5. Effect of DLX3 and ETS2 on *CGA* promoter.

The -255 *CGA* promoter – luc reporter was transiently transfected with ETS2 and DLX3 expression vectors, either alone, or in combination. Both DLX3 and ETS2 alone, modestly up-regulates the *CGA* promoter by 2.5 to 4 fold respectively. However, combination of DLX3 and ETS2 synergistically up-regulated the *CGA* promoter – luc reporter activity by 20 – 25 fold. Data from three independent experiments, each run in triplicates were analyzed statistically using one-way ANOVA.

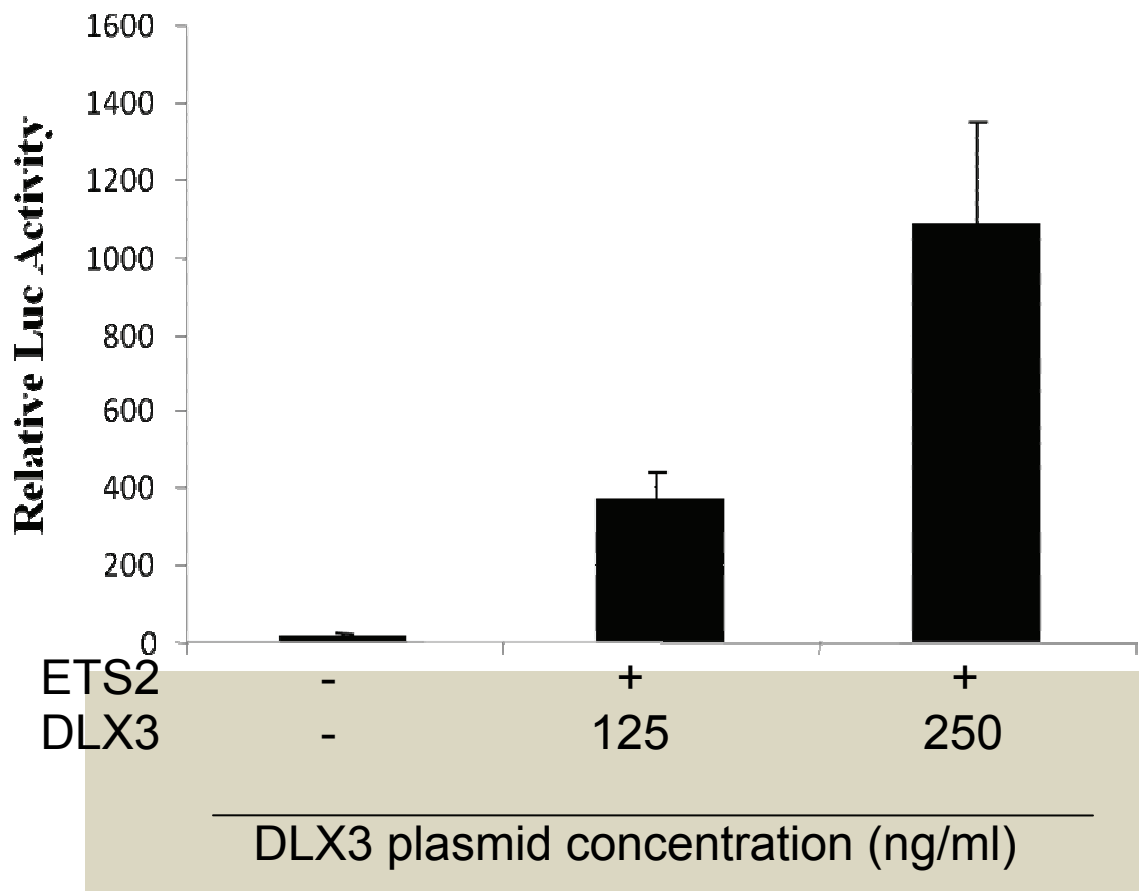
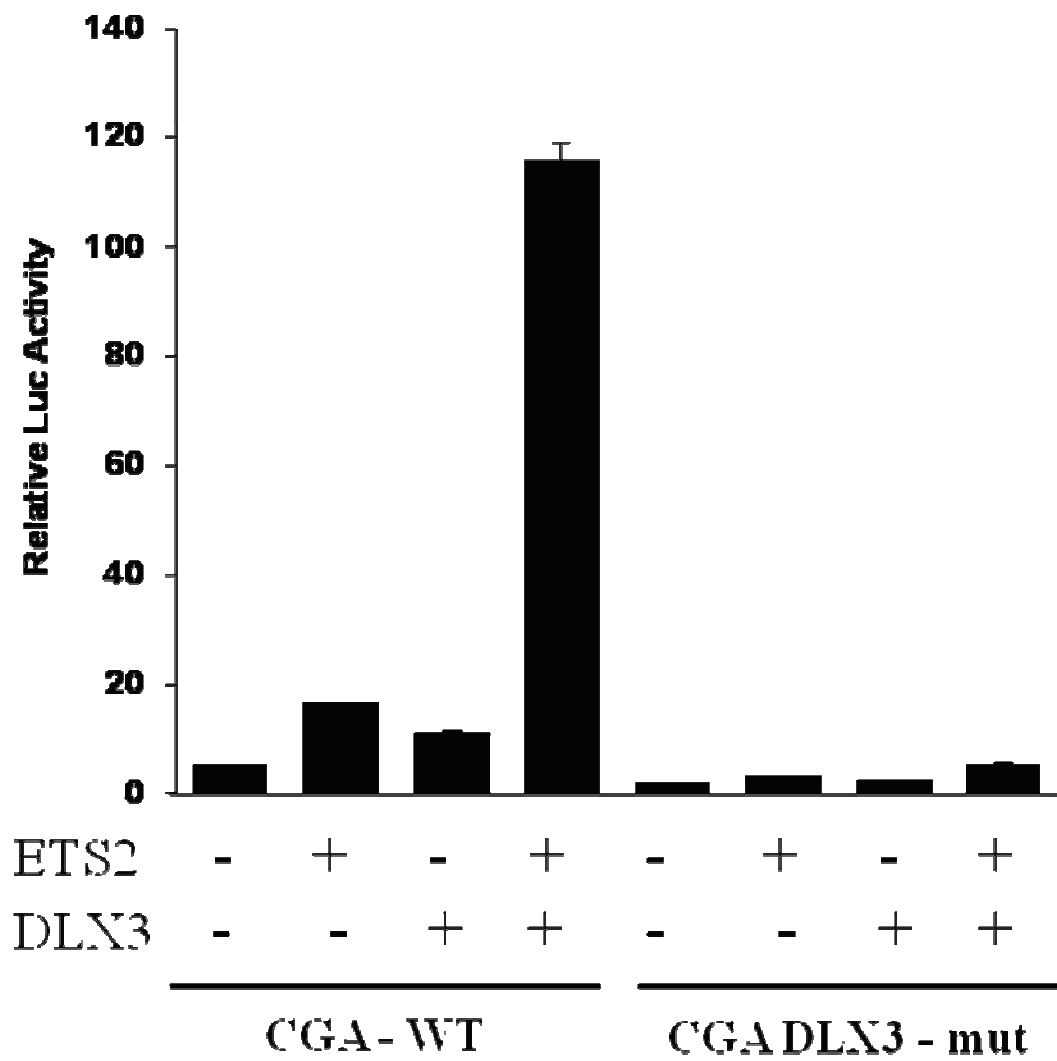


Fig. 6. Synergistic up regulation of *CGA* promoter by ETS2 and DLX3 is dose-dependent.

Transient transfection assay was performed with *CGA* – *luc* – reporter construct. Concentration of ETS2 expression vector was kept constant. Increasing concentration of DLX3 expression vector significantly increased the synergism.

A.



B.

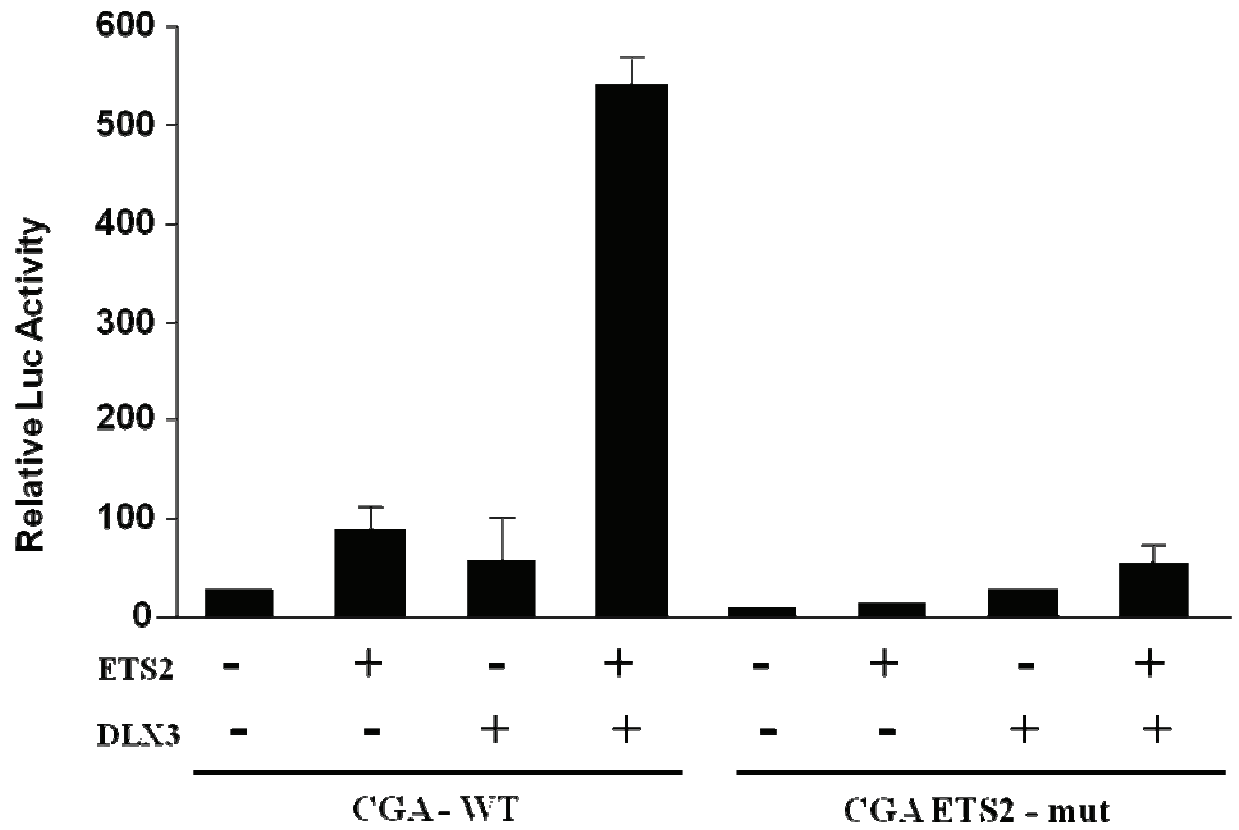


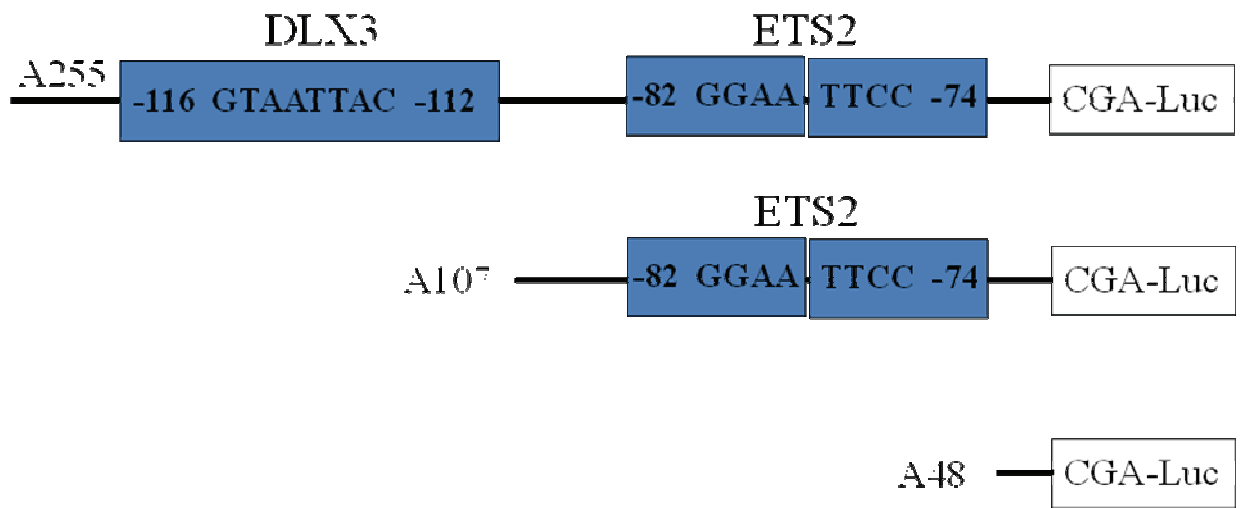
Fig. 7. Effect of DLX3 and ETS2 on wild-type *CGA* promoter as well as the *CGA* promoter with DLX3 binding site mutated.

(A) The -255 *CGA* WT promoter as well as the DLX3 – mutant promoter were transiently transfected with ETS2 and DLX3 expression vectors, either alone or in combination. Individually, DLX3 and ETS2 modestly up-regulate the *CGA* – WT promoter but fails to do so in the *CGA* DLX3 – *mut* promoter. Combination of DLX3 and ETS2 synergistically up-regulated the *CGA* – WT promoter activity. However, mutation of the DLX3 binding site completely abolished the synergism.

(B) Effect of combinatorial expression of DLX3 and ETS2 on wild-type and ETS2 – mutant *CGA* promoter.

In the wild-type promoter, DLX3 and ETS2 alone have a modest effect on transactivation. Combinatorial expression of DLX3 and ETS2 synergistically up-regulate the *CGA* – WT promoter activity. However, in the ETS2 – mutant promoter, the synergism is significantly reduced but not completely abolished.

A.



B.

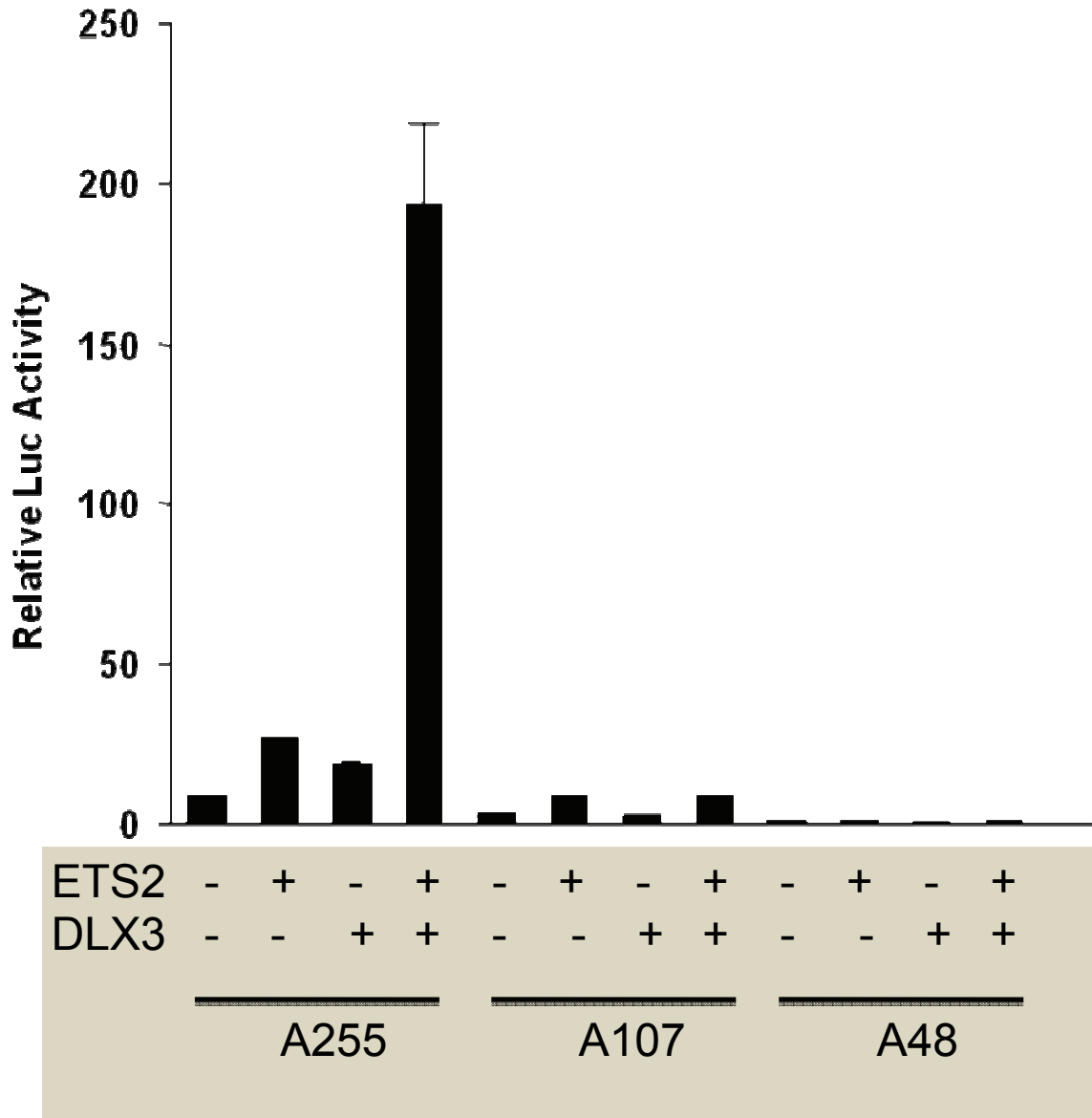


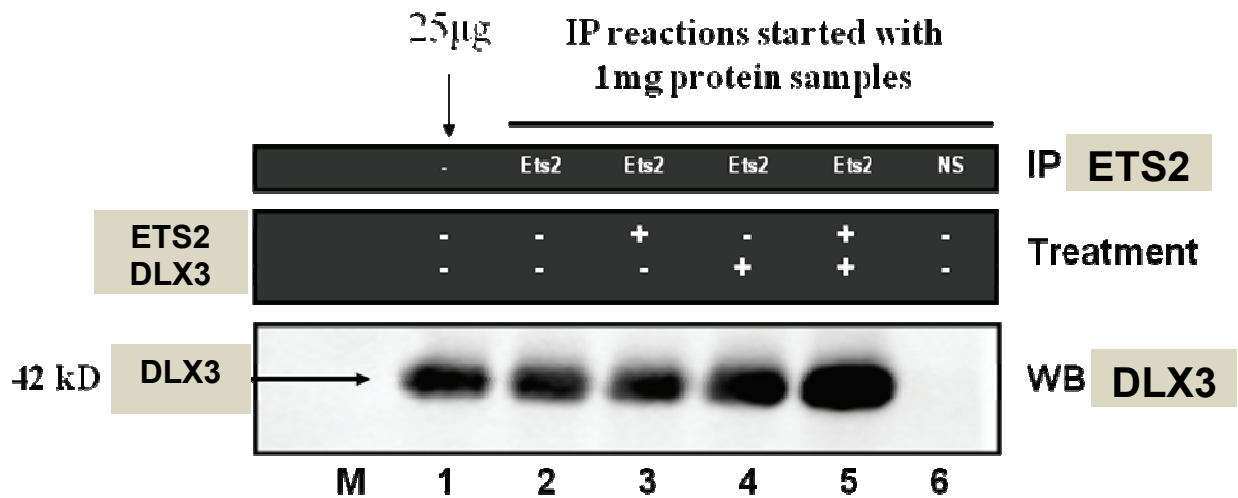
Fig. 8. Schematic diagram representing progressively deleted *CGA* – *Luc* reporter constructs.

(A) The full-length construct contains intact DLX3 and ETS2 binding sites. The shorter promoter lacks the DLX3 binding site only and the shortest promoter construct is devoid of either DLX3 or the ETS2 binding sites.

(B) Effect of combinatorial action of ETS2 and DLX3 on progressively deleted *CGA* promoter constructs.

DLX3 and ETS2 synergistically transactivates the *CGA* – 255 – *luc* reporter construct containing both DLX3 as well as ETS2 binding sites. The synergism was significantly reduced in *CGA* – 107 – *Luc* reporter construct that is devoid of the DLX3 binding site. On using the shortest promoter (*CGA* – 48 – *Luc*) that is devoid of both DLX3 as well as ETS2 binding sites, the synergism was completely abolished. The relative luc values of three independent experiments were log transformed.

A.



B.

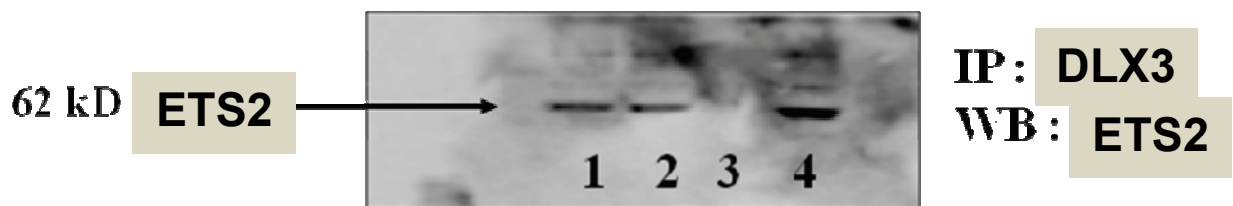


Fig.9. Co-immunoprecipitation analysis to determine any physical interaction between ETS2 and DLX3.

- A. IP was performed with affinity-purified ETS2 antibody and western analysis was performed with affinity-purified DLX3 antibody. 25 µg of the whole JAr cell lysate was loaded in the gel. Lane 2 is endogenous ETS2-DLX3 interaction. The band intensity remains almost unchanged on over expressing ETS2 (lane 3). However, over expression of DLX3 increased the band intensity a little bit, suggesting that endogenous level of DLX3 is limiting, or endogenous level of DLX3 is less compared to ETS2 (lane 4). On over expressing ETS2 and DLX3 together, the band intensity increase further (lane 5). Non-immune serum was used as a negative control (lane 6).
- B. Reverse coimmunoprecipitation analysis was also performed where the complex was immunoprecipitated with affinity-purified DLX3 antibody and western analysis with affinity-purified ETS2 antibody. Band intensity in the ETS2 and DLX3 over-expressed lysate was higher than the endogenous control.

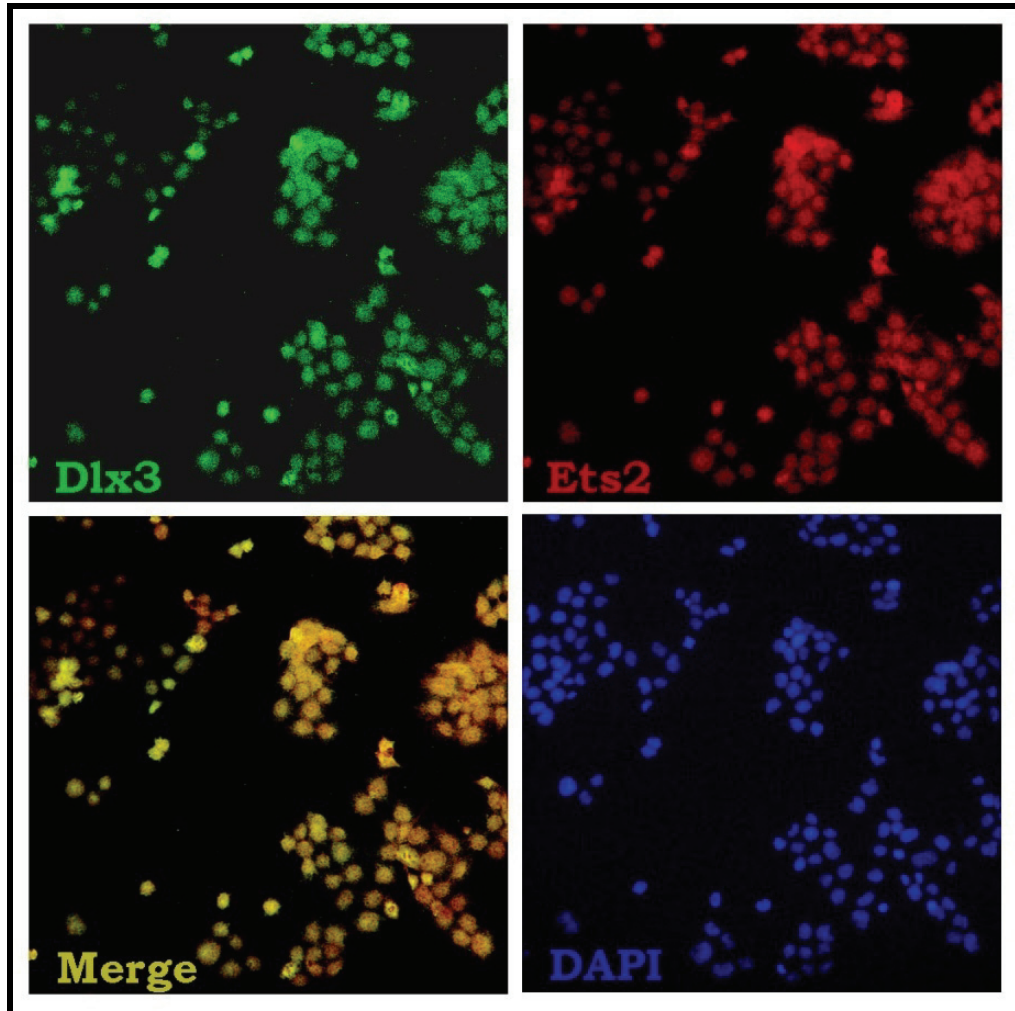


Fig.10. Immunofluorescence studies were performed by Dr. Padmalaya Das from our lab to determine whether ETS2 and DLX3 transcription factors co-localize in the JAr cells. DLX3 (green) and ETS2 (red) were demonstrated to colocalize in the JAr cell nucleus (merge in yellow). Nuclei were stained with DAPI.

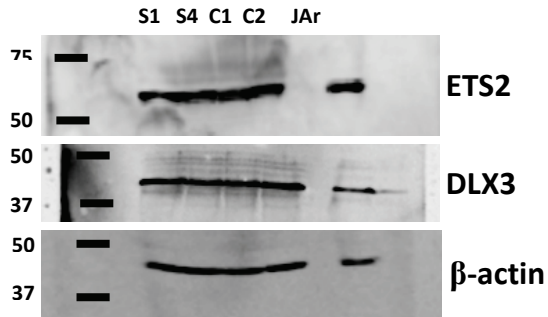
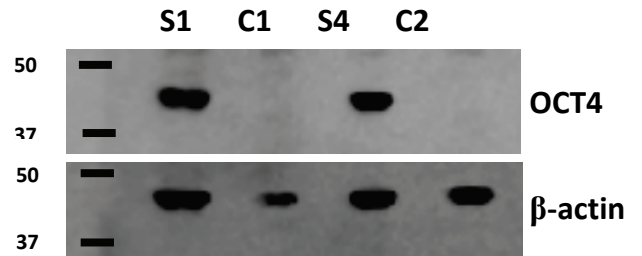
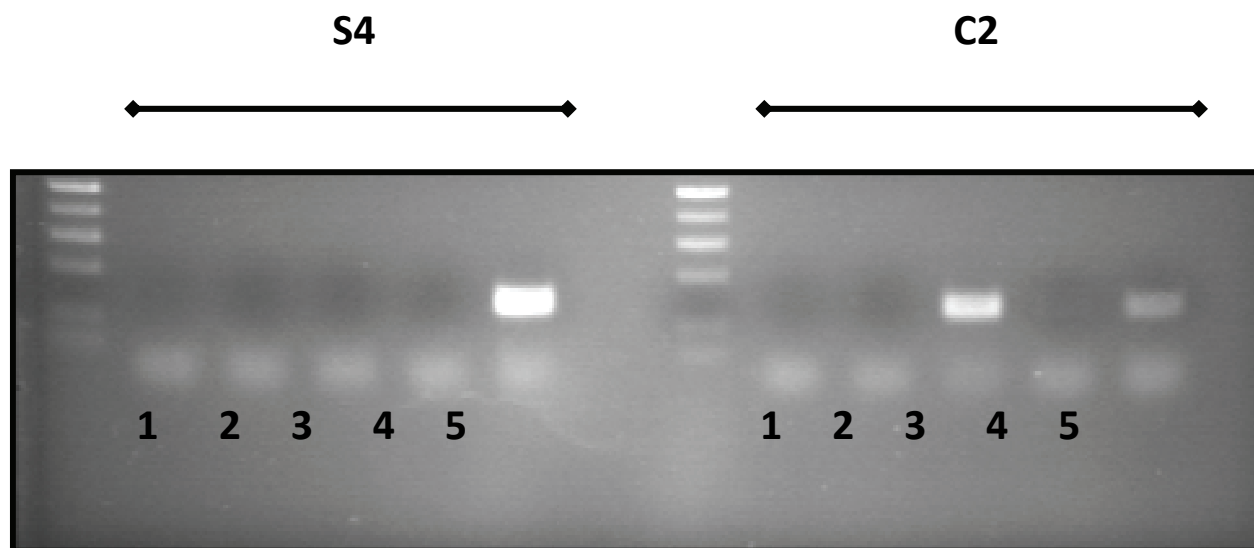
A.**B.**

Fig. 11. Expression of ETS2 and DLX3 proteins in presence or absence of OCT4. JAr S cells (S1 and S4) stably transfected with OCT4 expression vector as well as C cells (C1 and C2) stably transfected with pcDNA3 empty vectors were lysed and 30 μ g of crude cell lysates were analyzed by SDS-PAGE, followed by western analysis.

(A) ETS2 and DLX3 protein levels remained unchanged in S and C cells. Beta-actin was used as a loading control. Three separate western analyses were performed for fig. A.

(B) OCT4 protein expressions were restricted only to S1 and S4 cells. C1 and C2 control cells were OCT4 negative. Beta-actin was again used as a control. For fig. B, same blot was used to probe with three different antibodies.

A.



- 1: No antibody control
- 2: OCT4 antibody
- 3: ETS2 antibody
- 4: Non-specific IgG control
- 5: Total Input

B.

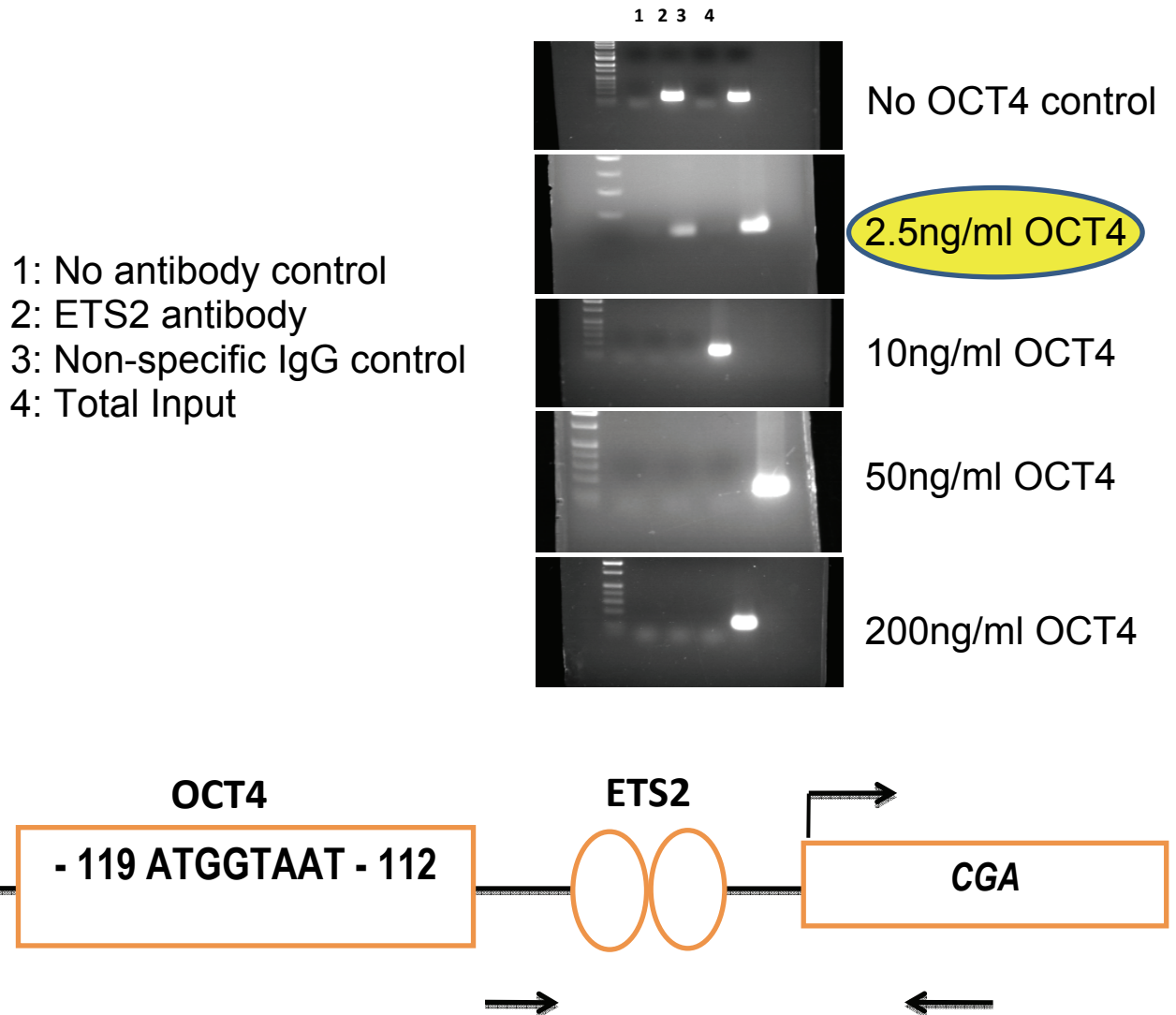


Fig.12. Association of ETS2 transcription factor with *CGA* promoter in absence of OCT4.

(A) Sheared chromatin prepared from JAr S4 and C2 cells were separately exposed to overnight incubation with no antibody control (lane 1), anti-OCT4 (lane 2), anti-ETS2 (lane 3), non-specific IgG (lane 4) and 10% of total input chromatin (lane 5). ETS2 failed to form complex with the promoter in presence of OCT4 (S4 chromatin). On the contrary, a strong ETS2-DNA complex was observed to form in absence of OCT4 (C2 chromatin).

(B) Sheared chromatin was prepared from normal JAr choriocarcinoma cells as well as JAr cells transfected with various concentrations of OCT4 expression vector. The sheared chromatin was exposed to no antibody control (lane 1), anti-ETS2 (lane 2), non-specific IgG (lane 3) and 10% of total input chromatin (lane 4). ETS2 formed a strong complex in absence of OCT4, but the complex was not formed even in presence of as low as 10ng/ml concentration of OCT4.

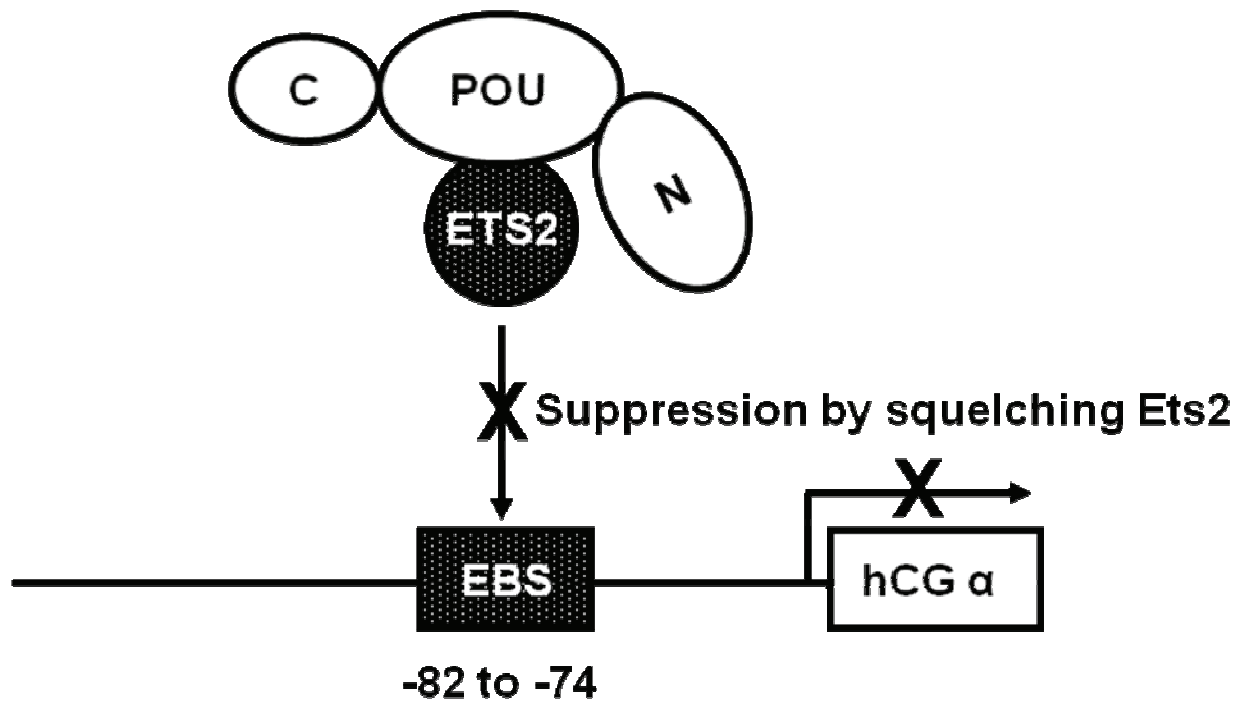


Fig.13. OCT4 silences *CGA* promoter activity by squelching ETS2. Schematic diagram representing binding of OCT4 to ETS2 and preventing the latter from transactivating the promoter.

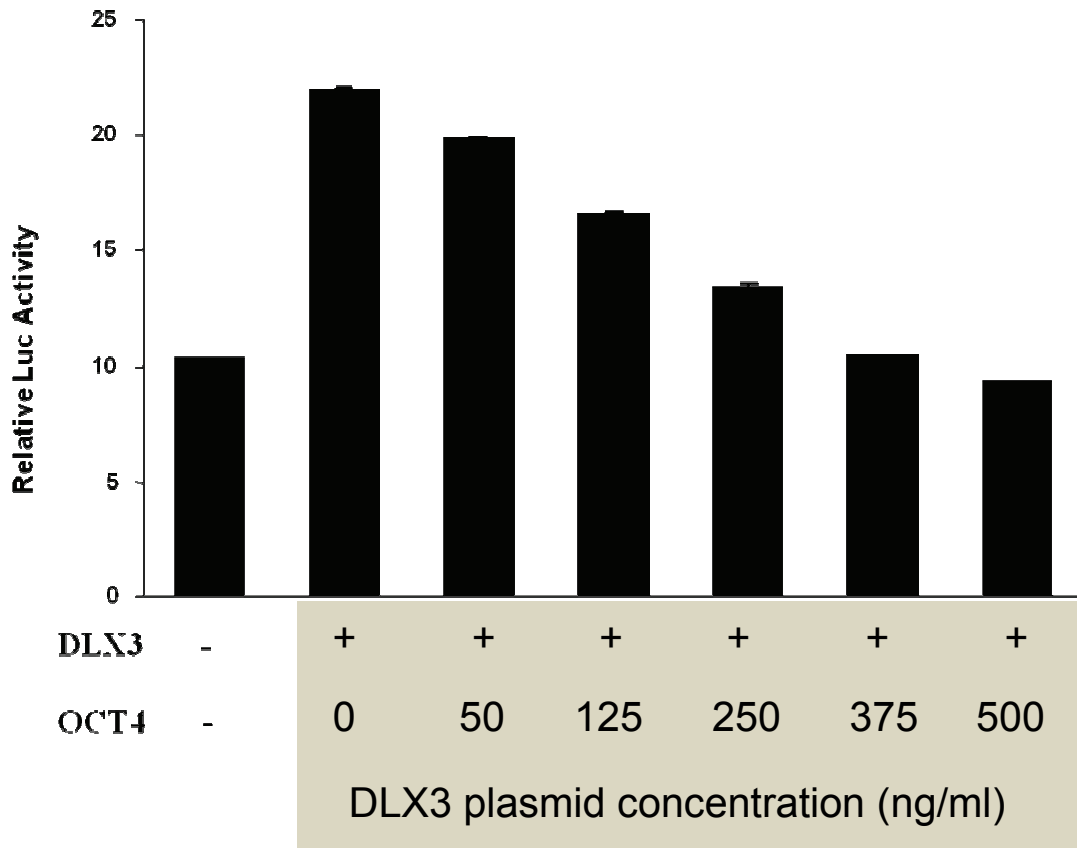


Fig 14. DLX3 partially interferes with OCT4 mediated repression of *CGA* promoter activity.

Expression of DLX3 alone modestly transactivates the *CGA* promoter activity. This transactivation was repressed in a dose-dependent manner by co-expression of OCT4. A significantly higher concentration of OCT4 expression vector (750 – 1000 ng) or (275 – 500 ng / ml) is required to reduce the DLX3 mediated transactivation of the *CGA* promoter by ~ 50 %. The relative luc activity values were log transformed.

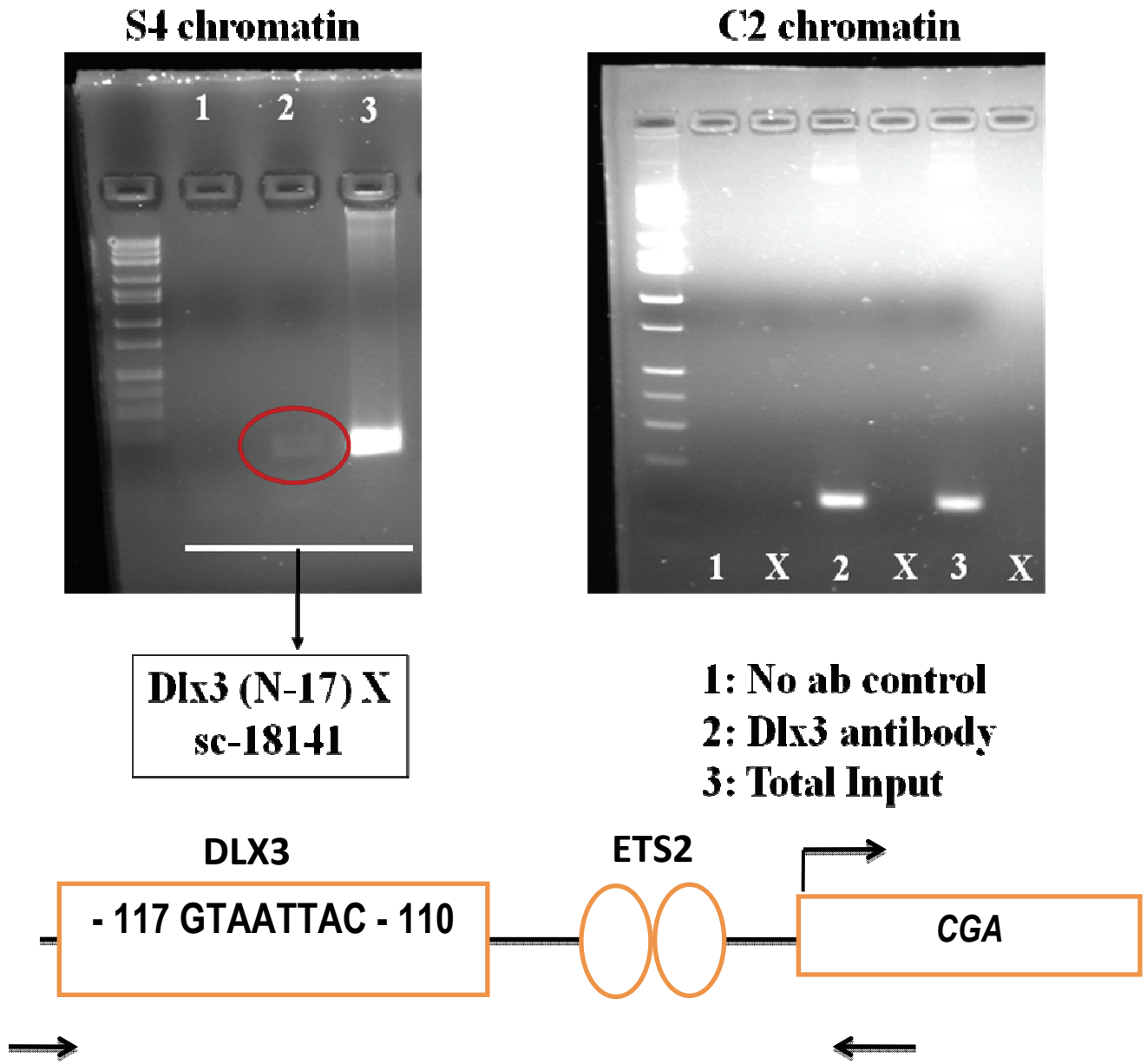


Fig. 15. Association of DLX3 transcription factor in absence of OCT4. Sheared chromatin prepared from JAr S4 and C2 cells were separately exposed to overnight incubation with no antibody control (lane 1), anti-DLX3 (lane 2) and 10% of total input chromatin (lane 3). DLX3 formed a strong complex with the DNA in absence of OCT4 (C2 chromatin). However, even in presence of higher concentration of endogenous OCT4 expression vector (S4 chromatin), formation of a DLX3 – DNA complex was significantly reduced, but not completely abolished (marked in red circle). Two different agarose gels were used to analyze the samples. Changes in band intensity of DLX3 were compared to its respective total input control band intensity.

Chapter III

Role of OCT4 alone in driving partial reprogramming of JAr choriocarcinoma cells towards a less differentiated phenotype.

Abstract:

OCT4 is a key transcription factor involved in maintenance of pluripotency and self-renewal in the inner cell mass and epiblast of embryos, embryonic germ cells and embryonic stem cells where a critical level of expression is required for maintenance of stem cell characteristics. It acts as an important molecular switch, so that alterations in its expression can alter cell fate. Recent studies have shown that forced expression of OCT4 along with NANOG, KLF4 and c-MYC in somatic cells from a variety of species, including human, can successfully lead to the induction of a pluripotent phenotype. Here, we investigated the properties of JAr choriocarcinoma cell lines developed in the mid-1990s that stably express OCT4 to determine whether these cells show features of de-differentiation. Microarray analysis performed on RNA isolated from two such cell lines were compared with controls, and the data confirmed by real time PCR on a select number of genes. A range of genes were differentially regulated by OCT4 expression, only a minority of which (~20 %) were down-regulated. Among these were *CGA*, *CGB* and *LHB*. The developmental pluripotency-associated genes (DPPA), *DPPA2* and *DPPA3*, were up-regulated by OCT4 expression, as were *CDX2*, *ZFP42 (REX1)*, *GATA2* and *KLF11*. The increased expression of *CDX2* was unexpected, as OCT4 and *CDX2* have been proposed to share a reciprocal relationship, which contributes to trophoblast emergence from pluripotent precursor cells. In general, however, the results were

consistent with the conclusion that the OCT4 expressing cells had reverted to a more stem cell-like phenotype.

Introduction:

A pluripotent cell is one that can generate any of the various kinds of cells that comprise the organism. Pluripotency, along with the capacity for continuous self-renewal, is considered to be a defining feature of ES cells. Pluripotency of a mammalian cell can be experimentally verified by its ability to contribute to embryonic development and to the generation of chimeras after being injected into a blastocyst (Jaenisch et al, 2008). Mouse and human ES cells have been extensively studied, but the features of these cells that cause them to be pluripotent are not fully understood. During the process of maintenance of pluripotency, the self-renewal process allows ES cells to duplicate themselves without undergoing differentiation (Xi et al, 2005). This self-renewal process can be achieved by symmetric cell divisions *in vitro* and asymmetric divisions *in vivo* (Xi et al, 2005). Under experimental conditions, pluripotency can be maintained by growing the ES cells in cultures supplemented with various kinds of growth factors including cytokines and LIF (in mouse) and BMP4 and FGFs (in humans) (Thompson et al, 1998). In mouse ESCs, LIF and IL-6 related cytokines signal through a common glycoprotein 130 (gp130) receptor (Burdon et al, 2002). LIF and gp130 recruit JAK kinases that permits the activation of STAT3 and mitogen-activated protein kinase (MAPK) pathways (Matsuda et al, 1999). Human ESCs can be routinely cultured on feeder fibroblasts, which supply additional growth factors such as activin, to maintain their undifferentiated state.

Recent advances in understanding the molecular mechanisms governing ES cell pluripotency and self-renewal have provided insights into the role played by various key transcription factors including OCT4, SOX2 and NANOG (Chambers et al, 2003; Nichols et al, 1998; Niwa et al, 2000; Mitsui et al, 2003) in maintaining the stemness phenotype. These same pluripotency factors, along with c_MYC, have also been shown to participate in reprogramming, a process by which differentiated cells can be induced to revert to a pluripotent state (Jaenisch et al, 2008; Egli et al, 2008), as first demonstrated for murine and human cells by using retroviral vectors designed to over-express the genes (Takahashi et al, 2006; Yu et al, 2007). OCT4 appears crucial to re-programming. It was originally described by Scholer et al (Scholer et al, 1990) as a member of the murine octamer – binding protein family and was associated with the ICM of embryos. It was speculated that the level of OCT4 expression might play a significant role in regulating the cell-fate during early embryonic development. In mouse embryos, OCT4 regulates cell-fate in a dose-dependent manner (Niwa et al, 2000). An increase in OCT4 expression drives differentiation towards primitive endoderm and mesoderm. By contrast,, repression of OCT4 drives differentiation towards trophectoderm. Only an optimal amount of OCT4 expression can sustain stem cell self-renewal (Niwa et al, 2000). These studies suggested the presence of a complex regulatory network in ES cells that tightly controlled OCT4 expression and ensured pluripotency. The discovery of NANOG offered a clear candidate for regulation of OCT4. NANOG is considered essential for maintenance of pluripotency and regulation of ICM/epiblast cells during embryonic development. Mouse embryos lacking NANOG fail to develop beyond blastocyst stage due to lack of epiblast (Mitsui et al, 2003). Its over-expression also prevents

differentiation towards primitive endoderm (Chambers et al, 2003). Another transcription factor, SOX2 exhibits a similar expression pattern to OCT4 during early stages of mouse embryo development, but, unlike OCT4, continues to be expressed in the trophectoderm. SOX2 is probably important for lineage specification and its deletion causes early post-implantation failure (Roberts et al, 2004). SOX2 also appears to partner with OCT4 in controlling gene expression in pluripotent cells (Rodda et al, 2005; Ambrosetti et al, 1997). Genome-wide studies on human ES cells identified more than ~ 1000 genes regulated by a combination of OCT4, NANOG, and SOX2 (Boyer et al, 2005). At least half of the genes that bound OCT4 to their promoter regions also bound SOX2, and the majority of these genes also had associated NANOG as well, implicating the importance of a strong inter-regulatory network of these three transcription factors in maintaining ES cell self-renewal. In other words, these factors silence various other transcription factors whose expression drives differentiation towards different lineages. In addition, quantitative chromatin immunoprecipitation analysis indicated that chromatin remodeling takes place close to or associated with OCT4 and NANOG binding sites to establish a conformation that is compatible with transcriptional activation (Freberg et al, 2007). Based on these large-scale data sets, it was proposed that OCT4, SOX2 and NANOG together are crucial for both self-renewal and pluripotency (Boyer et al, 2005). Apart from OCT4, SOX2 and NANOG, several other genes are characteristically up-regulated in pluripotent stem cells compared to normal somatic cells. Some of these same genes are also often over-expressed in tumors and include STAT3 (Niwa et al., 1998), E-RAS (Takahashi et al., 2003), c-MYC (Cartwright et al., 2005) and KLF4 (Li et al., 2005). Most likely these genes also contribute towards pluripotency and/or the long-term

maintenance of the ES cell phenotype as well as proliferative capacity of ES cells in culture.

As indicated above, reprogramming somatic cells by over-expressing certain “stemness” genes has recently allowed differentiated cells to become pluripotent. However, the first successful reprogramming of mammalian cell nuclei was demonstrated by cloning of sheep (Wilmut et al, 1997) and later other mammals. In these experiments, the donated somatic cell nucleus became re-programmed by factors present in the oocyte cytoplasm. Reprogramming has also been achieved by fusing somatic cells with ES cells. Thus factors common to oocytes and early embryos were likely candidates to test as candidates for driving de-differentiation. Takahashi and Yanamaka (Takahashi et al, 2007) initiated their experiments to re-program murine fibroblasts with a panel of genes, which they co-transfected in various combinations before settling on just four, namely *OCT4*, *SOX2*, *KLF4* and *c-MYC*. The resulting cells were quite similar in phenotype to murine ES cells and were shown to be pluripotent by a range of criteria, including the ability to contribute to chimeras and generate pups. Later, analogous procedures were employed to reprogram human fibroblasts to a pluripotent state by using the same or different combinations of transcription factors Takahashi et al, 2007; Huangfu et al, 2008b). Low efficiency of reprogramming has been overcome by using small molecular weight compounds, such as HDAC inhibitors (Huangfu et al, 2008a; Huangfu et al, 2008b). As these approaches were essentially empirical, there has been interest in defining the core molecular circuitry and determining which of these factors are absolutely essential in reprogramming to a pluripotent state. To date, OCT4 has been employed in all reprogramming experiments, either as a transfected gene or as a protein.

In this chapter, I utilized JAr choriocarcinoma cells stably transfected with OCT4 expression vector in which the open reading frame of murine OCT4 was driven by CMV promoter under the selection of G418. These cell-lines were developed over a decade ago in our laboratory (Liu et al, 1997) with the expectation that certain signature genes, especially CGA and CGB would be down-regulated, thereby mimicking effects of transiently-expressed OCT4 on reporter genes under control of *CGA* and *CGB* promoters (Liu et al, 1996; Liu et al, 1997). Here my goal was to extend the analysis to other genes and to determine whether OCT4 expression caused the cells to revert to a less differentiated state through partial reprogramming.

Materials and methods:

Cell culture:

JAr cells (HTB-144; American Type Culture Collection) and JAr cells stably transfected either with pcDNA3-OCT4 (clones S1 and S4) or with pcDNA3 alone (clones C1 and C2) (Liu et al, 1996; Liu et al, 1997) were maintained in 90% DMEM with 10% fetal bovine serum.

RNA extraction and preparation for microarray analysis:

RNA was isolated from the control JAr cells and four JAr cell derivative lines (S1, S4, C1 and C2) by using RNA STAT-60 reagent (Tel-Test Inc, Friendswood, TX). All JAr cells were cultured in parallel and passaged at the same time. RNA samples (n = 3 per line) were prepared from approximately 6×10^6 cells from each cell line at successive cell passages. Sample amplification, labeling and hybridization on Illumina HumanWG-6

Sentrix BeadChips were performed according to the manufacturer's instructions by using Illumina Bead-Studio software (University of Missouri DNA core facility). Each sample was hybridized to an individual chip, for a total of 15 chips.

Microarray Data Analysis:

The gene expression analysis was performed with the Illumina BeadStudio software. Four groups of experimental data (C1 C2, S1, and S4) with three independent biological replicates per group were analyzed. The data were then normalized by using the Rank Invariant method. The Differential Expression for each probe was then assessed by using the Illumina Custom Expression model for pair-wise groups. This analysis provided a *p*-value for each probe for each pair-wise comparison. Details can be found in BeadStudio Gene Expression Module: User Guide, Illumina Doc #11207533 Rev. A, Chapter 4.

Quantitative real-time PCR analysis:

A selection of the differentially expressed genes obtained from the microarray analysis was validated by quantitative real-time PCR analysis by using Sybr Green Mastermix (Applied Biosciences, Branchburg, NJ). Primer express software (Applied Biosciences) was used to design the primers (Table 2).

Western blot analysis:

Whole cell lysates were prepared from the stably transfected JAr lines S1, S4, C1 and C2 cells by using RadioImmunoPrecipitation Assay (RIPA) buffer containing 50 mM

Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 0.1 % SDS (w/v), 1 % Triton X-100 (w/v) and 1X protease inhibitor cocktail (Sigma, St. Louis, MO). Lysates were centrifuged (4000 x g) to remove particulate matter. Cleared cell lysates were analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Pre-stained dual label protein ladders (BioRad) were used as molecular weight markers. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA). Affinity purified rabbit anti-CDX2 polyclonal antibody (Anaspec Laboratories, Fremont CA) was diluted 1:20000 and rabbit anti-OCT4 (raised in our laboratory) was diluted 1:1000 before use. Rabbit anti- β -actin antibody (Cell Signaling Technologies, Danvers, MA) was diluted 1:1000. Secondary antibodies used were HRP-linked anti-sheep IgG or HRP-linked anti-rabbit IgG (Cell Signaling Technologies, Danvers, MA) diluted 1:5000. All antibody dilutions were made in 5 % non-fat dry milk (NFDM) (w/v). Membranes were developed with Photo-type Horseradish Peroxidase western blot detection system (Cell Signaling Technologies). Images were acquired with the Fuji LAS 3000 imaging system (Fujifilm Medical Systems, Stamford, CT). Some of the blots were stripped with Restore-Plus Western Blot stripping buffer (Thermo Scientific, Rockford, IL), blocked with 5% non-fat dry milk (w/v) and re-probed with a different antibody. The Manufacturer's protocol provided by Thermo Scientific was followed.

Immunofluorescence studies:

JAr cells, stably transfected with OCT4 expression vector (S4 cells), as well as the control cell-lines (C2 cells), were grown on cover-slips in 6-well plates (35 mm dishes).

Each cell-line was treated in duplicate along with one secondary antibody control only. The monolayer of cells was fixed with 2 % paraformaldehyde (PFA), followed by permeabilization with Triton X-100. Blocking was performed with 5 % bovine serum albumin (BSA) (w/v) and 5 % goat serum (w/v) for 30 min. Overnight incubation with affinity purified rabbit anti - OCT4 antibody (1:100 dilution) and affinity purified chicken anti – CDX2 antibody (1:100 dilution) (both antibodies were affinity purified in our laboratory) was performed at 4 °C. The control wells were incubated with BSA only. Secondary antibodies AF – 568 (red) for OCT4 and AF – 488 (green) for CDX2 were both diluted 1:50 2 h in dark. DAPI staining was performed for 15 min, followed by mounting the coverslips on glass slides. Immunofluorescence was observed under a fluorescent microscope (Model: Olympus).

Results:

Differential regulation of genes by OCT4:

The microarray analysis revealed a relatively high number of genes that were differentially regulated by cells expressing OCT4 compared to the cells that had been transfected with the vector alone (the control cells). Importantly, OCT4 itself was up-regulated, although its expression in the two S-cell populations was still relatively modest. The genes are listed based on increasing p -values starting with as low as 0.00004 (TABLE 2). Around 150 genes were differentially regulated ($P < 0.05$). The differential score indicates difference between the C average signal and S average signal. A differential score with a positive value indicate genes that were up-regulated. On the other hand, down-regulated genes had a differential score with a negative value. Of the

top 100 genes regulated by OCT4, only 21 showed reduced expression compared to the control cell-lines.

Down-regulated genes: As expected from the earlier work of Liu et al (Liu and Roberts, 1996; Liu Leaman et al, 1997), *CGA* and *CGB* were down-regulated in the S cells over-expressing OCT4. Both *CGB5* and *CGB8* were similarly down-regulated. Interestingly, *LHB* was also expressed in JAr cells, suggesting that JAr cells likely produce hLH as well as hCG. In addition, *STAT5*, which is considered as an early marker of differentiation (Nemetz and Hocke, 2002) was also significantly down-regulated ($P < 0.001$). G-protein coupled receptor 1 (*GPCR1*), leucine rich repeat *NALP1*, ryanodine receptor *RYR2*, F-box protein 32 (*FBXO32*), and histidine decarboxylase (*HDC*) were among other genes significantly down-regulated in the OCT4-expressing cells.

Up-regulated genes: Among the top 100 regulated genes, 79 genes demonstrated increased expression. Up-regulation of *DPPA2* and *DPPA3* suggests that OCT4 alone can partially reprogram differentiated JAr cells. Among the other genes up-regulated, included *ZFP42 (REX1)*, *GATA* factors, *KLF11*, *CDX1* and *CDX2*. Previous microarray analysis demonstrated that OCT4 positively regulates *ZFP42* expression (Babaie, Herwig et al. 2007). Our data is consistent with those data. Up-regulation of *CDX2* was surprising in the sense that previous studies have demonstrated that first cell-fate choice in mammalian embryo, the segregation of ICM and trophectoderm is regulated by mutually antagonistic effects of OCT4 and *CDX2* transcription factors (Strumpf, Mao et al. 2005). Previous studies on reprogramming of somatic cells gave indicated the necessity of a group of transcription factors that included OCT4, SOX2, NANOG and c-MYC (Papapetrou, Tomishima et al. 2009). Later, it was demonstrated that Kruppel – like –

factors such as KLF2, KLF4 and KLF5 are required for the self-renewal of ES cells (Jiang, Chan et al. 2008). In our microarray analysis, one of the members of KLF family, KLF 11 showed up among top 100 genes that were differentially regulated by OCT4. OCT4 significantly ($P < 0.001$) up-regulated *KLF11* expression. Regulation of few of these genes has also been validated by quantitative real-time PCR analysis.

In addition to the expression of these genes, expression pattern of *CGA*, *CGB* as well as *LHB* were also validated. Micro-array results indicate that percentage of reduction in expression was highest in *CGB*, followed by *LHB* and finally *CGA*. However, the quantitative real-time PCR data significantly deviated from the previous ones. Both *CGA* and *CGB* were significantly down-regulated as opposed to a moderately reduced expression of *LHB* (Fig. 3).

CDX2 and OCT4 protein expression in S and C JAr cells:

Immunofluorescence localization and western blotting experiments were performed to confirm that OCT4 protein was expressed in the S, but not in the C cell-lines and that CDX2 was up-regulated in response to OCT4 in the S cells (column 2, row 1). Nuclei of almost all the S4 cell population but not of any C2 cells were positive for OCT4. CDX2, on the other hand, was expressed in both S4 and C2 cell-lines (column 1, row 1 and 3). The secondary antibody controls (where no primary antibody was used) (S4/C and C2/C) showed no positive fluorescence above background (row 2 and 4).

Western blotting confirmed that OCT4 is expressed in only the S1 and S4 cells and not in the C1 and C2 cell-lines (Fig. 2). CDX2, as expected, was expressed in all four cell-lines. The band intensity of CDX2 whole cell lysates from the S1 and S4 cells seemed to be

slightly higher than in C1 and C2 cell-lines but this comparable with the β -actin loading controls too.

Discussion:

Early work investigating the downstream targets of OCT4 in embryos identified a number of genes that appeared to be transcriptionally regulated, including that for the growth factor *FGF4* (Kunath et al 2007; Rodda et al 2005) and the transcription factor *ZFP42* (Babaie, Herwig et al. 2007). The first report of OCT4 acting as a negative regulator of gene expression was from this laboratory when it was noted that stable expression of OCT4 in JAr choriocarcinoma cells markedly down-regulated the production of hCGA (Liu, Leaman et al. 1997) and hCGB (Liu and Roberts 1996) protein production and mRNA expression. Recent genome-wide studies have identified many additional down-stream targets of OCT4 that are up-regulated especially ones that encode self-renewal factors, i.e. also called “stemness” or pluripotency genes (Boyer, Lee et al. 2005; Babaie, Herwig et al. 2007), lineage-specific factors, and signaling molecules (Scholer, Dressler et al. 1990; Guo, Costa et al. 2002) as well as genes involved in DNA damage repair (Campbell et al 2007). Genes that are negatively regulated by OCT4 in embryonic stem cells (in combination with NANOG and SOX2) are frequently transcription factors that control lineage specification pathways (Rossant 2004). Thus, OCT4 is implicated in a broad spectrum of cellular processes that collectively specify the self-renewal state and pluripotency of ES cells and prevent differentiation along one or more of the embryonic germ layers. In addition, a characteristic of all reports in which the somatic cells have been re-programmed to pluripotency is a requirement for OCT4

(Kuroda and Tada 2006; Loh, Wu et al. 2006). Indeed, OCT4 alone has been demonstrated to be sufficient to induce pluripotency in adult neural stem cells (NSCs) (Kim, Habiba et al. 2009).

Our first hypothesis was that several genes, in addition to *CGA* and *CGB*, especially ones controlled by *ETS2*, would be silenced by OCT4, presumably by a squelching mechanism. Accordingly, use was made of cell-lines developed in this laboratory almost 15 years ago in which OCT4 expression had been up-regulated in an attempt to revert these cells to a less differentiated state in which some signature genes of differentiated trophoblast might not be expressed. These cells do indeed express OCT4 mRNA and protein (Table 2 and Fig. 2), while control cells that had been transfected with the vector without an inserted cDNA did not. Almost the entire S4 cell population were OCT4 positive (Fig. 1), but despite the presence of OCT4, only a relatively small number of genes were down-regulated in S cells. Apart from the anticipated effect on *CGA* and *CGB5 & 8* and the structurally related *LHB*, there was a significant down-regulation of *SDK2*, *STAT5B*, *WNT3*, as well as select genes belonging to solute carrier family and zinc finger protein family. Whereas *STAT5B* and *WNT3* control normal mammalian growth and development, as well as enhancing hematopoietic differentiation of embryonic stem cells (Boiani and Scholer 2005), it would appear, therefore, that OCT4 expression did cause the JAr cells to become “less differentiated”. Nevertheless, few genes became strongly down-regulated and the microarray data indicated only relatively modest effects on transcript levels. These outcomes were surprising, as general effects on *ETS2* – regulated genes were anticipated. Various matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) that are involved in human placental

invasiveness have been previously demonstrated to be regulated by ETS2 (Vincenti 2008; Vu and Werb, 2000; Cohen et al, 2006). From our microarray data, the *MMPs* and *TIMPs* were modestly down-regulated ($P > 0.5$). It is possible that the effects were less than dramatic because OCT4 is expressed only weakly in these genetically modified JAr cells compared to pluripotent cells. Conceivably, OCT4 mRNA and protein are unstable in the somatic cell background or the T7 promoter (used to drive expression) is only weakly active.

Importantly, the up-regulation of various DPPAs including *DPPA 2, 3 & 4* clearly indicates the potentiality of OCT4 alone in driving partial dedifferentiation. In mouse, *Dppa2 & 4* (both closely-linked SAP DNA-binding motif genes) are responsible for maintenance of pluripotency in ESCs (Maldonado-Saldivia et al, 2007). Moreover, the Takahashi et al group has demonstrated that induction of pluripotent stem cells from adult human fibroblast by defined transcription factors clearly increased the expression of *DPPA 2 & DPPA 4* genes (Takahashi et al, 2007). *DPPA 3*, also known as *STELLA* or *PGC7*, has been demonstrated as another member playing a significant role in maintaining self-renewal in ESCs (Bowles et al, 2003). *ZFP42 (REXI)* was also significantly up-regulated ($P < 0.01$) in JAr cells expressing OCT4. Others have shown that *REXI* is a down-stream target of OCT4 and is under positive regulation (Yuan, Corbi et al. 1995; Niwa 2001; Catena, Tiveron et al. 2004). *REXI* is also regarded as one of the important marker genes of undifferentiated, pluripotent stem cells (Hosler, LaRosa et al. 1989) and has been identified as essential for ES cell self-renewal in both mouse (Chen, Wu et al. 2007) as well as in human (Mongan, Martin et al. 2006) ESC. Our microarray

data are consistent with a role for OCT4 in regulating these genes, and suggest that the S-cell lines have undergone a degree of de-differentiation.

One surprising observation was that *CDX2* expression was increased in the presence of OCT4. Previous studies have demonstrated that the first cell fate choice in the mammalian embryo, namely the segregation of ICM and trophectoderm, is apparently regulated by the mutually antagonistic effect of OCT4 and CDX2 transcription factors (Strumpf, Mao et al. 2005), although this view must now be modified in view of the fact that NANOG is also involved (Medvedev, Shevchenko et al. 2008). CDX2 has been proposed to be required for repression of ICM specific genes such as *OCT4* and *NANOG* in the trophectoderm, while OCT4 represses *CDX2* expression in emerging trophectoderm. Either repression of OCT4 or over-expression of CDX2 can cause differentiation of mouse pluripotent cells towards trophectoderm (Niwa, Toyooka et al. 2005), while *CDX2* knock-out prevents the maturation of trophectoderm in mouse blastocysts (Meissner and Jaenisch 2005). Finally, expression of *CDX2* is a feature of mouse trophoblast stem cells (Niwa, Toyooka et al. 2005; Tolkunova, Cavaleri et al. 2006). During mouse development, CDX2 begins to be expressed relatively early in development but becomes localized mainly to outer blastomeres in morulae and to the trophectoderm in blastocysts (Eda, Osawa et al. 2002). OCT4, on the other hand, is expressed throughout the early embryo until the late blastocyst stage, when expression becomes restricted to the ICM and developing epiblast (Pesce and Scholer 2001; Boiani, Eckardt et al. 2002). *Oct4* *-/-* mutant mice die around the time of implantation and only trophectoderm-like cells can be recovered, suggesting the importance of *Oct4* in specification of ICM (Nichols, Zevnik et al. 1998). Overall, therefore, the up-regulation

of *CDX2* in the S-cells was puzzling, although it is consistent with partial reprogramming of trophoblast cells towards their stem cell origins.

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Table 1. Synthetic oligonucleotides used for real-time PCR analysis

Name	Forward primer	Reverse primer
KLF11	TGCCGGGAATACCAAGTTGT	AGTTCCTCCTTCGGGAAAAGT
DPPA3	CGAATCTGTTTCCCCTCTATCG	CTCTCCTGCTGTAAAGCCACTC
GATA2	TTAACAGGCCACTGACCATGAA	TTTGACAGCTCCTCGAAGCA
ZFP42	TGGGCCTTATGTGATGGCTAT	TCACCCCTTATGACGCATTCT
18S	TTCGGAAGTGGAGCCATGAT	TTTCGCTCTGGTCCGTCTTG
OCT4	GCAGCTCAGCCTTAAGAACATG	TCTCATTGTTCGGCTTCCT
CGA	TGCCCAATACTTCAGTGCAT	TCTGAGGTGACGTTCTTTTGGGA
DPPA2	CTTTGCGGGACTGGTGTCAT	CTTGCCGTTTCAGGGTAAG
CGB	TCAGCTGTCAATGTGCACTCTG	TTGAGGAAGAGGAGTCCTGGA
CDX2	AAGTGTCCCAGAGCCCTTGA	AGGGACAGAGCCAGACACTGA
LHB	CCGCAGCACCTCTGACTGT	TAGAGGAAGAGGAGGCCTGA

Table 2. List of genes differentially regulated by OCT4

Average signals of S and C cells show fold up-regulation or down-regulation. The differential score is a number (+ or -) that reflects comparison between average C values and average S values. Av. C value less than av. S value = + diff.score (up-regulated). Av. C value greater than av. S value = - diff.score (down-regulated). Among top 100 genes, only 20% are down regulated (data not shown).

GENE NAME	s.Diff Pval	s.DiffScore	c.AVG Signal	s.AVG Signal	s. STDEV
DPPA2	0	61.235	114	243.6	29.251
CGB8	0	-92.469	134.6	34.1	17.091
CGB5	0	-77.991	158.1	39.1	14.438
CGB	0	-69.069	107	33.7	12.742
LHB	0	-100.668	285.7	100.4	30.737
EVPL	0.00004	43.839	172.1	307.1	62.048
POU5f1	0.00012	39.241	18.3	59.8	13.858
SERPIN6	0.0002	37.047	1250.2	2071.1	479.072
EAF2	0.00089	30.502	93.9	164.6	213.917
DDC	0.00097	30.151	70.6	125.8	37.937
ABHD8	0.00104	29.829	94.4	166.4	17.71
SDK2	0.00114	-29.423	126.5	34	44.926
STAT5B	0.00115	-29.379	152.6	92.5	31.567
SLC35D2	0.00163	-27.885	464.9	311.4	13.503
SLC19A3	0.00193	27.138	162	1107.2	56.263
WNT3	0.0021	-26.781	366.2	215	724.666
CCKBR	0.00268	25.712	1375.3	2156.5	59.342
DPPA3	0.00278	25.553	1127.7	2949.9	53.205
LTBP4	0.00282	25.491	115.8	184.7	471.363
SHRM	0.00342	24.664	46	99.1	1126.746
ZNF462	0.00407	23.904	18.9	75.1	23.736
TM7SF2	0.00423	23.736	613.7	883.5	63.981
DNMT3L	0.00474	23.242	5403.7	7807.7	33.928
NALP1	0.006	-22.222	149.2	41.3	42.447
ADARB1	0.00625	22.039	84.9	133.9	180.527
TCEAL7	0.00627	22.03	238.6	346.9	286.56
PLTP	0.0066	21.803	2650.7	3701.2	1114.319
ZNF273	0.00684	21.65	187.5	274.3	16.285
RABGAP1L	0.0071	21.486	38.3	70.3	23.077
ZFP42	0.00746	21.272	2262.7	3143.9	61.878
CDX2	0.00816	20.882	8.2	54.1	31.018
PBX4	0.00886	20.527	19.5	46.2	383.268
SLC7A8	0.009	20.459	218.8	448.6	44.685
PAWR	0.00908	20.417	3699.7	5091.7	14.688

SART2	0.01024	19.896	639.2	881.4	619.784
APAF1	0.01056	19.761	892.1	1289.2	36.369
TBC1D9	0.011	19.586	324.9	451.2	275.442
DOPEY2	0.01177	19.293	147.4	210.6	37.382
MORN3	0.01292	18.886	582	796.1	12.565
CGA	0.01332	-18.756	6972.5	4617.3	203.178
EIF1AY	0.0139	18.57	2779.5	3754.5	380.594
ZHX1	0.01439	18.42	1096.4	1482.9	38.852
CEACAM1	0.01467	18.334	225	379.7	187.046
DUSP3	0.01588	17.991	1421.4	2037.6	29.121
ADCY9	0.0168	17.748	347.8	472.5	161.076
ZC3HAV1	0.01747	-17.578	535.8	195.3	99.431
SGK3	0.01785	17.483	156.7	218.7	66.894
GSTO1	0.01947	-17.105	713.7	505.6	80.052
GPR1	0.0199	-17.012	128.5	70.9	37.036
UBTD1	0.02093	16.792	118	176.6	173.823
PTD004	0.0215	-16.676	54.4	27.3	1806.902
DEGS1	0.02206	16.564	768.5	1031.5	334.25
FBXL3	0.02254	16.47	299.2	405.7	210.817
CHAF1A	0.023	16.383	358.7	479.6	144.561
IFRG15	0.02412	16.176	122.3	170.4	416.592
ADAM19	0.02447	16.114	1926.8	2577.8	10.299
HPS5	0.02646	15.774	304.9	406.1	526.85
RYR2	0.02667	-15.74	72.5	43.3	95.433
CNNM2	0.02749	15.609	57	86.6	59.404
RPS6KA1	0.02754	15.6	739.6	971.7	44.727
MCM10	0.02766	15.581	935.7	1233.2	121.43
CDR2L	0.02772	15.572	98.9	141	149.151
ZNF91	0.02824	15.492	235.7	315	79.485
SIRT4	0.02827	15.486	158.4	215.1	21.21
RAB40A	0.02847	15.456	201.1	270.6	20.92
SALL4	0.02858	15.44	1442	2629.9	52.575
CDX1	0.02861	15.435	-3.1	26.2	8.695
FBXO32	0.02866	-15.427	221	132.3	113.725
SPATS2	0.02872	15.418	265.3	353	93.882
PELI1	0.02915	15.354	140.1	199.7	33.756
SOX15	0.02934	15.326	689	1094.4	24.702
SNAI1	0.0296	15.287	176.2	240.8	78.553
NEB	0.02966	15.278	53.8	91.9	53.158
CSH1	0.02995	15.235	112	239.8	39.217
SUNC1	0.03002	15.226	410.6	1272.4	437.352
HDC	0.03026	-15.191	161.5	2.7	62.467
ADARB1	0.03055	15.15	1019.7	1536.1	14.477

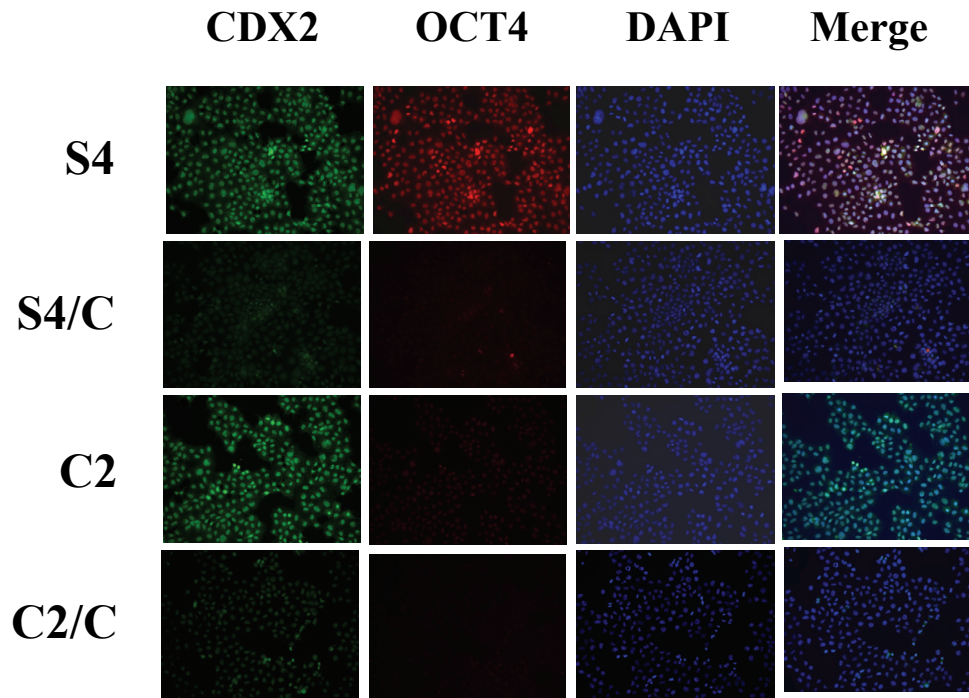


Fig.1. Immunofluorescence localization of CDX2 and OCT4 in JAr S4 and C2 cell lines. In brief, these two cell lines were grown on coverslips in 35mm dishes. The cells were fixed with 2% paraformaldehyde (PFA), permeablized with Triton X-100 and blocked with 5% bovine serum albumin (BSA) and 5% goat serum. Following blocking, primary incubation was performed with OCT4 and CDX2 antibodies (mentioned in detail in the materials and methods section). Primary incubation was followed by washing steps and secondary incubation with respective alexa fluor (AF) tags. Nuclear staining was performed with DAPI. The slides were mounted and sealed on glass slides and observed by microscopy.

S4 cells stained positive for OCT4 (Red, Row 1) as well CDX2 (Green, Row 1). S4/C is the secondary control only for S4 cells (Row 2). The C2 cells stained positive for CDX2 (Green, Row 3) but negative for OCT4 (Red, Row 3). C2/C is the secondary control only for the C2 cells. In the S4 and C2 controls, the cells were incubated with only secondary AF-tagged antibodies. Specific localization to nucleus was confirmed by staining with DAPI (Blue).

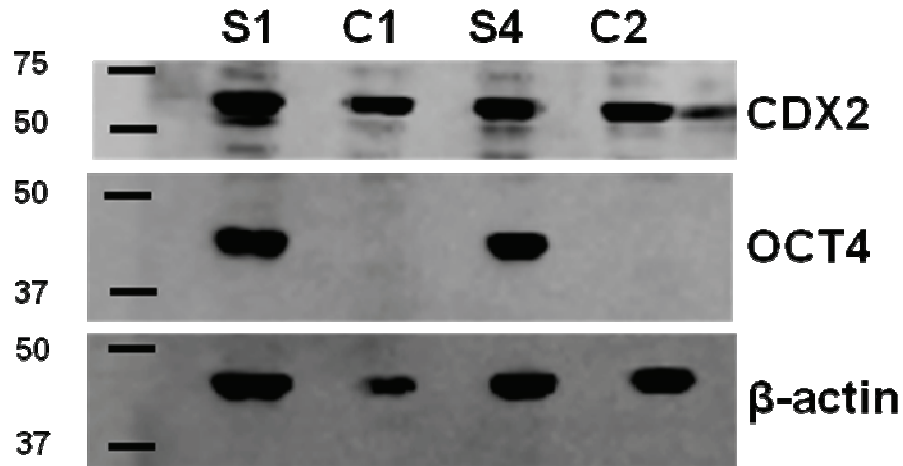
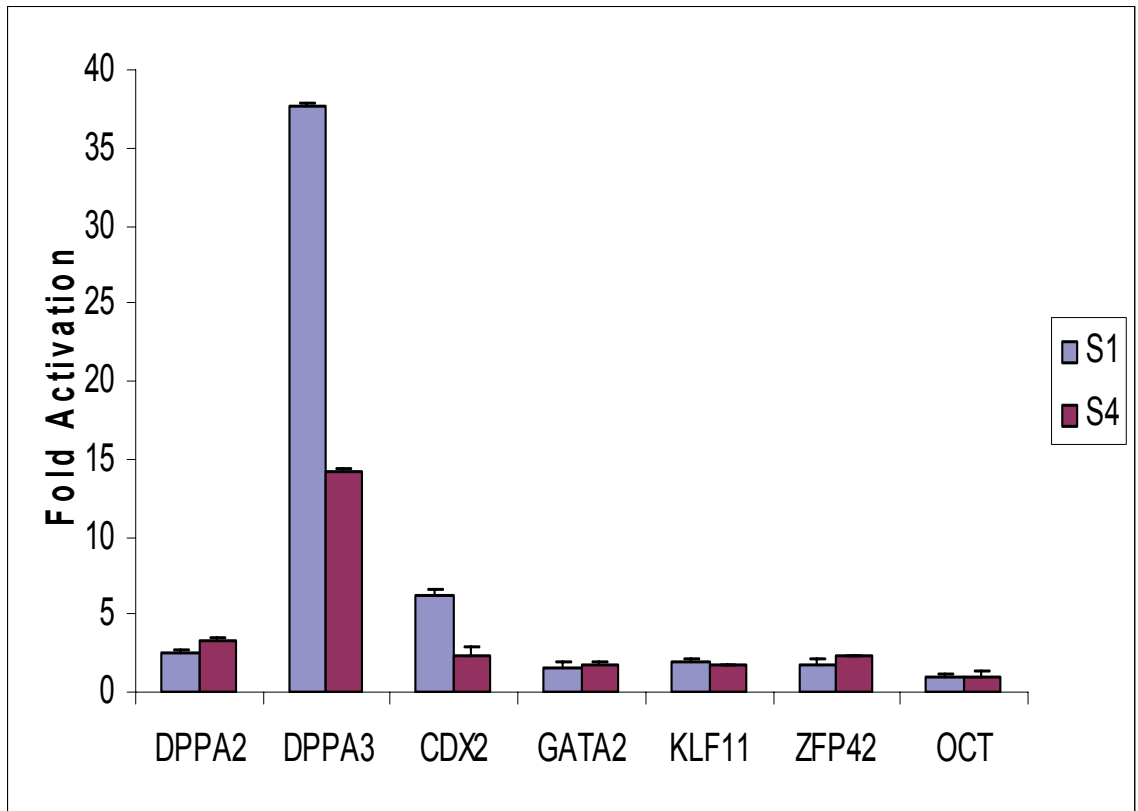


Fig.2. Western blotting demonstrates expression of CDX2 and OCT4 proteins in S1, S4, C1 and C2 cells.

60 μ g of protein (whole cell lysate) was loaded in each well and analyzed in 12.5 % SDS-PAGE gel. Proteins were transferred to PVDF membranes, blocked with 5% NFDM and incubated with CDX2 antibody. Primary incubation was followed by secondary incubation with HRP – conjugated secondary antibodies. Blot was developed using FUJI Imaging system. For CDX2 blot, an additional band was observed in the last lane although it was empty. It might be a minor spillage from loading C2 cell extract. The same blot was stripped and incubated with OCT4 antibody as well as control beta –actin antibody (details mentioned in the materials and methods).

A.



B.

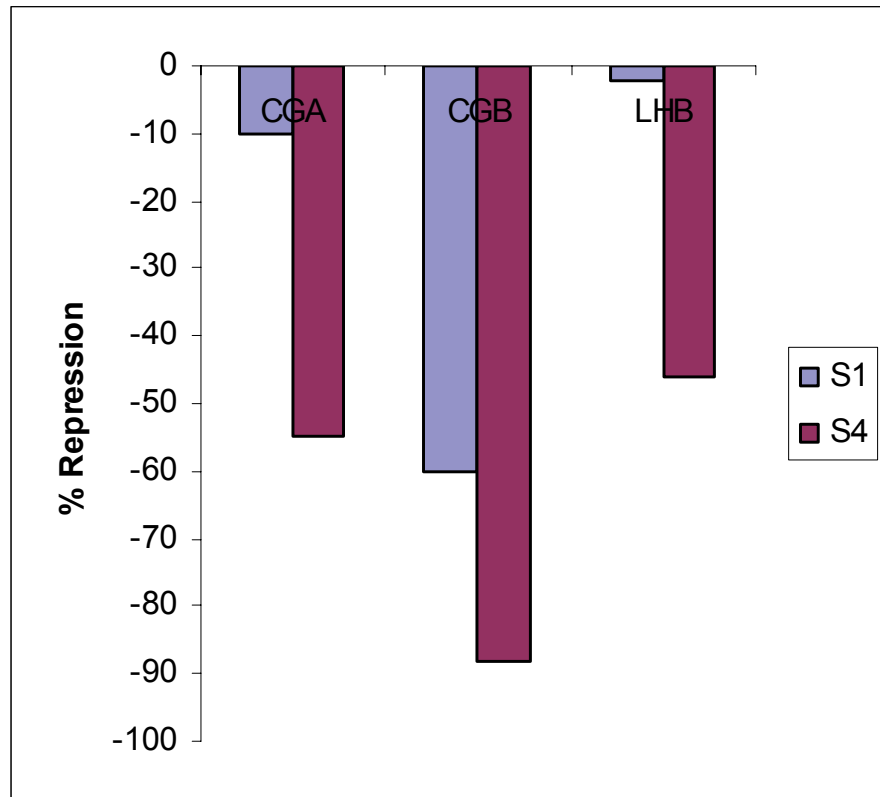


Fig. 3. Real-time PCR data analysis to validate the differential regulation of a few genes by OCT4.

- (A) *DPPA2*, *DPPA3*, *OCT4*, *CDX2*, *ZFP42*, *GATA1* and *KLF11* are up-regulated by OCT4. These data indicate good correspondence between the S1 and S4 cells among most of the genes. However, a few of these genes, including *DPPA3* and *CDX2* are highly expressed in S1 as opposed to S4 cell-lines. These data are consistent with the microarray results. The Y-axis represents the fold activation of these genes generated by comparing the fluorescence threshold (Ct) values of the sample of interest (S1 and S4) with a control (C1 and C2) samples or a calibrator. Each experiment has been repeated at least 2 to 3 times and the data represented here are the average values.
- (B) *CGA*, *CGB* and *LHB* were among the top 100 genes that were down-regulated by OCT4. The Y-axis represents percent repression of these genes in S1 and S4 cell lines. These data interpretations deviated a little from the microarray data that showed higher percentage of LHB repression than in real-time.

VITA

Rangan Gupta was born in Calcutta, West Bengal, India on August 04, 1978. He completed his secondary education from Nava Nalanda High School in 1995 and higher secondary education from Jodhpur park Boys school in 1997. He received his Bachelor of Science degree in Microbiology from University of Calcutta in 2000. He remained in Calcutta and received his Master of Science degree in Molecular Biology, Biophysics & Genetics from University of Calcutta in 2002. He completed his internship training on Pharmacokinetics & Pharmacogenomics from Astra Zeneca India based at Bangalore. He came to United States of America in the year 2003 and started his doctoral (graduate) training on a cell-cycle regulation project. Next year, in June 2004, he moved over to a different lab and started working on a different project on Human Chorionic Gonadotropin gene regulation and finally completed his doctorate of philosophy in the fall of 2009.