Human chorionic gonadotropin (hCG), is a member of glycoprotein family hormone which also comprises luteinizing hormone (LH), follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH). It belongs to the cystine knot family of growth factors (Isaacs, 1995). hCG is initially secreted by the pre-implantation embryo and subsequently by the trophoblast. It is responsible for rescue of the corpus luteum, thereby sustaining progesterone secretion until placental progesterone synthesis commences.

hCG is a heterodimeric molecule consisting of an α-subunit non-covalently associated with a hormone specific β-subunit. The isolated individual subunits are devoid of hormonal activity (Catt et al., 1973). The α-subunit is common to hCG, LH, FSH and TSH and contains 92 amino acids. The β-subunit of hCG (βhCG), which confers biological specificity, contains 145 amino acids including a 24 amino acid C-terminal extension lacking in βLH. The homology between the β-chains of hCG and LH is about 80% and hCG and LH exert their action through the same receptor. The molecular weights of hCG, αhCG and βhCG are 36.7, 14.5 and 22.2 kDa respectively.

Although hCG is normally produced in significant amounts only during pregnancy, it is also ectopically made by trophoblastic as well as non-trophoblastic (e.g. colon, prostate, bladder, breast and lung) carcinomas (Braunstein et al., 1973; Hattori et al., 1978; Blackman et al., 1980; Marcillac et al., 1992; Nishimura et al., 1998; Bellet et al., 1997). βhCG has therefore been proposed as cancer marker of broad utility (Cole and Butler, 1998; Regelson, 1995; Acevedo et al., 1995). In general, hCG and/or subunit synthesis is seen in poorly differentiated tumors (Lazar et al., 1995). hCG expression is prominent in metastatic cancers and is associated with poor prognosis (Acevedo & Hartsock, 1996; Cole et al., 2006). Survival time of patients with hCG-expressing cervical, pancreatic and colorectal cancers has been reported to be statistically shorter than those with hCG negative neoplasm (Crawford et al., 1998, Syrigos et al., 1998). In bladder cancer as well, the serum level of hCG is considered a prognostic indicator of disease (Gillott et al., 1996). The presence of hCG has been linked to chemo- (Kuroda et al., 1998) and radio- (Butler et al. 2000) resistance and the molecule has been shown to increase invasiveness (Zygmunt et al., 1998).
Several lines of evidence point to the potential role of the molecule in the establishment, maintenance and progression of neoplastic disease. It has been suggested that ectopically produced hCG (or its subunits) may exhibit immunosuppressive properties, thereby permitting metastasis (Cole et al., 2006; Iles, 2007), or that it may have growth factor-like properties for tumor cells (Gillott et al., 1996; Cole and Butler, 2008).

Evidence demonstrating an autocrine role for hCG subunits has been gathered for lung cancer cells; ChaGo human lung tumor cells lose their characteristic anchorage-independent growth phenotype in the presence of anti-α-hCG antibodies. In addition, cells transfected with anti-sense α oligonucleotides display several altered phenotypic properties and exhibit reduced growth, accompanied by loss of anchorage-independence and tumorigenicity in nude mice (Rivera et al., 1989). Anti-hCG antibodies can induce the killing of uterine cervix adenocarcinoma cells (Kalantarov and Acevedo., 1998). Passive administration of anti-hCG antibodies causes tumor regression in an in vivo animal model system (Kumar et al., 1992).

Immunization against hCG thus may be an attractive option for cancer therapy. Although induction of hCG-specific cytotoxic T cells may be the end-goal in some forms of such approaches, it is expected that an hCG vaccine able to elicit and maintain effective levels of neutralizing antibodies would be able to reduce the growth or control metastasis at least in some types of gonadotropin-responsive cancers. A vaccine using the carboxy-terminal portion (CTP) of βhCG coupled to DT increased survival in patients with metastatic colorectal cancer (Moulton et al., 2000). Vaccine targeting β-Hcg have been found to be more immunogenic than the CTP formulation. One such vaccine developed as an anti-fertility option, has undergone Phase I clinical trials in human volunteers without causing any notable adverse effects (Singh et al., 1989; Talwar et al., 1990; Kharat et al. 1990). Antibodies were capable of inactivating hCG bioactivity (Pal et al., 1990; Pal and Singh, 2001). Phase II efficacy trials in women established the feasibility of the approach in preventing pregnancy (Talwar et al., 1994; Singh et al., 1994).
These studies reveal that it is indeed possible to induce bioeffective anti-hCG responses in human. The data reported in this thesis helps delineate the many ways in which hCG helps cancers grow and metastasize, thereby contributing to poor patient prognosis. In addition, using both in vitro and in vivo model systems and active and passive immunization protocols, demonstrates the potential utility of anti-hCG antibodies in the treatment of tumors that may be otherwise hard to treat due to acquired chemo-resistance.
The pregnancy hormone hCG is secreted by the placental trophoblast, with serum levels peaking in the first trimester of pregnancy. The primary function of hCG is to induce the production of progesterone and estrogen by the corpus luteum during early pregnancy. It also stimulates the development of the fetal testes (Hertig, 1968; Beard, 1902).

**History**

In 1912, Aschner injected water-soluble extracts of the human placenta into guinea pigs and studied responses of the genital tract (Aschner, 1912). In 1913, Fellner induced ovulation in immature rabbits with similar extracts (Fellner, 1913). In 1919, Hirose demonstrated the induction of ovulation and normal luteal function in immature rabbits repeatedly injected with human placental tissue (Hirose, 1919). These studies served to establish the hormonal link between the placenta and the uterus. In 1927, Ascheim and Zondek demonstrated that pregnant women produce a gonad-stimulating substance (Aschheim and Zondek, 1927). The name “human chorionic gonadotropin” has the following derivation: “Chorion” comes from the Latin word “chordate” meaning “afterbirth”, while “gonadotropin” refers to the fact that the hormone is a trophic factor for the gonads, acting on the ovaries to promote steroid production.

**Structure of hCG**

Both subunits of hCG contain a high content of mannose. The two subunits are intimately associated with each other, with each subunit having similar folds with two hairpin loops at one end and a single loop at the other (Wu et al., 1994). The assembly of hCG in the rough endoplasmic reticulum (RER) is made by threading the glycosylated end of hCGα loop 2 beneath a hole formed in a disulfide-latched strand of the β-subunit named the “seatbelt” (Xing et al., 2004). The carboxyl-terminal peptide (CTP) of hCGβ is in contact with hCGα in the native dimer and forms a seatbelt around the hCGα (residues β93–110) that stabilizes the heterodimer. The final closing of the β26–110 bridge locks the seatbelt and secures the αβ dimer, preventing disassembly (Ruddon et al., 1996). Folding and assembly of the subunits are dependent on the carbohydrate moieties and their specific positions on the peptide chains. The oligosaccharide branches from Asn-52 and Asn-78 assures normal secretion of hCGα and its removal reduces correct α-β dimerization (Matzuk and
Boime, 1988; Feng et al., 1995). The Asn-30 oligosaccharide on hCGβ is important for secretion but not assembly, and the Asn-13 branch mainly influences assembly (Fares, 2006). The polypeptide sequences α27–40 and α38–42 have an important role in dimer formation. Whereas αTyr-37 is a critical residue, αTyr-65 is involved in holding both subunits in the native conformation. The residues β90–111 wrap around a helical loop of the hCGα and play an important role in subunit assembly, but the whole hCGβ-CTP (β115–145) extension is not required for subunit association. Besides the sugar branches and the polypeptide composition of α and β subunits, the final conformation of the hCG dimer is stabilized by disulfide bonds. All non-cysteine residues within the hCG cystine knot are required for the formation and assembly of the dimer. Disulfide bonds α7–α31, α59–87 and α10–32 are not essential for the hCGα combination with the hCGβ, but the hCGβ disulfide bonds β9–57, β34–88 and β38–90 are essential for heterodimer formation (Mishra et al., 2003). The disulfide bond β26–110 is formed only after α-β assembly (Huth et al., 1992) (Fig 1.1). The cystine knot, with three disulfide bonds, creates a ring that includes the intervening polypeptide backbone, and a third bond penetrates this ring (Fig. 1.2).

Figure 1.1: Assembly of α and β subunits to form the hCG dimer. Thick red line, hCGα polypeptide chains; thick green line, hCGβ polypeptide chains; numbers refer to amino acid numbers; yellow and grey lines, disulfide bonds; thick black line, seatbelt region of hCGβ;
thick blue lines, seatbelt disulfide bonds; thin blue line, small loop disulfide bond of the β seatbelt (Xing et al. 2001).

**Figure 1.2:** Spatial representation of α-β assembly in which the hCGα carboxyl terminal extension penetrates the hCGβ and is locked by the β-seatbelt portion.

**Forms of hCG**

Multiple variants of hCG have been detected in serum and urine samples (Figure 1.3). Free hCGα and hCGβ have been demonstrated in serum of pregnant women, in the serum and urine of cancer patients and are also secreted by cancer cell lines. (Franchimont et al., 1978 and Cole et al., 1983). A terminal degradation variant of hCG β-subunit is found in urine samples. This is referred to as the β-subunit core fragment; it comprises of two fragments of β-subunit, β6–40 and β55–92, held together by disulfide bonds (Figure 1.3) (Amr et al., 1984). Urinary β-core fragment is used as a general tumor marker for all non-gestational malignancies (Butler, 2000; Regelson, 1995; Iles, 2007, Butler et al., 2003 and Iles, 1995). A larger, hyperglycosylated form of the hCG dimer is synthesized by choriocarcinoma cells (Swaminathan and Bahl, 1970; Elliott, 1997 and Ruddon et al., 1981); N-linked oligosaccharides are 1.5-fold larger and O-linked oligosaccharides 2-fold larger (Figure 1.3) (Elliott, 1997; Kobata and Takeuchi, 1999 and Valmu, 2006). Similar, unduly large N- and O-linked oligosaccharides have been demonstrated on the hCG free β produced by non-gestational cancer cells (Ruddon et al., 1981 and Muyan and Boime, 1997). Similar large oligosaccharides are found on the free α-subunit secreted in pregnancy (Blithe and Nisula, 1985). The free α-subunit can be further glycosylated in malignancy, gaining an additional O-linked oligosaccharide (Cole et al., 1984).

Degradation pathways for both regular and hyperglycosylated hCG involves elastase and other proteases which are secreted by either tumor-associated or circulatory macrophages (Cole et al., 1991). These enzymes cleave or nick hCG at β-subunit amino acids 44–45 or 47–48 (Elliott, 1997 and Cole et al., 1991) generating nicked regular hCG, nicked hyperglycosylated hCG, nicked hCG free β and nicked hyperglycosylated hCG free β (Figure 1.4). The enzymes further cleave and release the CTP on the nicked molecules (βCTP, β residues 93–145) generating nicked hCG missing βCTP, nicked hyperglycosylated hCG missing βCTP, nicked hCG free β missing βCTP and nicked hyperglycosylated free β missing βCTP (Elliott, 1997; Cole
et al., 2006 and Cole et al., 1991). All these variants are found in serum and urine samples in normal or abnormal pregnancies, gestational trophoblastic diseases or non-gestational malignancies (Figure 1.3).

Figure 1.3: Outline of the structures of the 15 common hCG variants present in serum and urine samples in either pregnancy, gestational trophoblastic disease or other malignancy.
Numbers refer to subunit polypeptide amino acid numbers (as in 1 and 145 in the 145 amino acid long β-subunit), O refers to O-linked and N to N-linked oligosaccharides. OO and NN refer to large or hyperglycosylated oligosaccharides. α refers to the α-subunit and β the β-subunit. βCTP is the C-terminal segment (residues 93–145) on the regular or hyperglycosylated hCG β-subunit.

Gene sequences for hCG were identified in the 1980s. A single-gene on chromosome 6 encodes the α-subunit protein of hCG. The β-subunit of hCG is encoded by six highly homologous and structurally similar genes (CGB1, CGB2, CGB3, CGB5, CGB7 and CGB8). These are arranged in tandem and inverted pairs on chromosome 19q13.3 (Fig. 1.4).

**Figure 1.4:** A diagrammatic representation of the arrangement of genes in the LHβ/hCGβ gene cluster on chromosome 19q13.32. The red arrows show the postulated chance stimulation of hCGβ-subunit genes by the GnRH promoting LHβ-subunit gene transcription.

**Physiology of hCG**

The essential role of hCG in pregnancy sustenance is supported by the observation
that administration of hCG antisera leads to termination of pregnancy (Stevens, 1975) and that the risk of miscarriage is correlated with low hCG levels (Dziechciowski and Klimek, 2001). Placental hCG replaces pituitary LH in controlling progesterone production at the initiation of pregnancy, from implantation (~3 weeks gestation) to 6 weeks of gestation. Progesterone synthesis by cells comprising the syncytiotrophoblast is independent of hCG stimulation and occurs from 6 weeks of gestation until term. Serum concentrations of hCG rise logarithmically; in early pregnancy, hCG levels usually double every 48 to 72 hours, and a concentration of 25 mIU/ml is considered diagnostic of pregnancy. Peak hCG levels are achieved at 10 weeks of gestation (Table 1), after which they fall to about one-fifth of peak levels, where they remain till term (Steinman and Cohn, 1973) With the decrease of hCG secretion, the placenta begins to secrete large quantities of estrogen and progesterone, and the sustenance of pregnancy is no longer dependent on the corpus luteum (Fig 1.5).

While it is clear that the primary role of hCG in early pregnancy is to extend the functional life of the corpus luteum in early pregnancy, its role in mid and late pregnancy remains a matter of debate (Keay et al., 2004; Illingworth et al., 1990). The fact that a variety of cells bear hCG receptors points to a broader physiological role. For example, through its receptors on the endometrial cells, hCG may promote smooth muscle relaxation and myometrial vasodilation, thus enabling implantation of the embryo. Via its receptors on trophoblast cells, hCG can stimulate progesterone production. hCG has also been demonstrated to stimulate cytotrophoblasts to secrete vascular endothelial growth factor (VEGF) indicating a role in early placental angiogenesis (Keay et al., 2004). hCG is also believed to mediate several biological functions which are critical to efficient placentation in humans. Firstly, hCG maintains angiogenesis in the myometrial spiral arteries through the duration of pregnancy, acting via LH/hCG receptors on the spiral arteries (Lie et al., 1992; Herr et al., 2007; Zygmunt et al., 2003; Zygmunt et al., 2002; Toth et al., 2002; Rao and Alsip, 2001). Secondly, hCG promotes the fusion of villous cytotrophoblast cells to syncytiotrophoblast (Cole et al, 2006; Shi et al, 1993). Further, Leukemia Inhibitory Factor, low levels of which have been implicated with infertility, is up regulated by hCG (Perrier et al., 2004)). Lastly, hCG also promotes the decidualization of stromal cells (Tang and Gurpide, 1993).
Figure 1.5: The levels of various hormones during pregnancy.

Table 1. hCG concentrations during pregnancy.
hCG also plays an important immunoregulatory role; it inhibits the production of IL-2 in peripheral blood mononuclear cells (PBMC) and modulates the immune response during pregnancy (Schafer et al., 1992). It suppresses mitogen-induced responses of T (Han, 1974; Kaye and Jones, 1971 and Adcock et al., 1973) and B (Hammarstrom et al., 1979) lymphocytes. hCG is reported to attract macrophages to the fetal–maternal interface, which is believed to reduce the exposure of maternal immune system to paternal antigens in the placenta (Wan et al., 2007; Schumacher et al., 2009).

**The hCG/LH receptor**

Protein hormones like hCG and LH weigh more than 5,000 Dalton, and therefore cannot act on cells without using membrane receptors. LH and hCG share a common receptor (Jacobsen and Henriques, 1992). Genetic analysis has revealed that exon 10 of the LH receptor could be responsible for discriminating between the LH and the hCG receptor (Keay et al., 2004). The hCG/LH receptor is located on corpus luteal cells of the ovary for promotion of progesterone, on the decidua for initial communication with the blastocyst on myometrial tissue for growth in line with fetus and for muscle relaxation, on uterine vasculature for angiogenesis, on umbilical cord tissue for growth, on fetal organs for growth and differentiation, on cytotrophoblast cells for differentiation, and on human brain cells, leading to hyperemesis gravidarum. As discussed above, hCG/LH receptors are present on several cells and tissues, and mediate a wide array of biological responses. The hCG/LH receptor responds to hCG, LH and hyperglycosylated hCG, but not hCG free subunits or nicked hCG (Cole, 2009). Evidence suggest that the α-subunit plays a prominent role in receptor binding while the β-subunit is responsible for receptor specificity (Rao, 1979; Dufau and Kusuda, 1987). The human hCG/LH receptor comprises 675 amino acids (Jia, 1991; Segaloff and Ascoli, 1993 and McFarland, 1989). The gene for the hCG/LH receptor is located on human chromosome 2p21, and contains 11 exons and 10 introns (Ascoli, et al., 2002; Fanelli and Puett, 2002; Fanelli, 2001); exons 1-10 and a portion of exon 11 encode the extracellular domain (Puett et al., 2005). hCG/LH receptor sequence and cloning studies indicate that it is part of a large family of guanine nucleotide binding protein (G-protein) membrane coupled receptors (McFarland, 1989 and Loosfelt, 1989).
**hCG signalling**

Stimulation of the hCG/LH receptor by hCG or LH activates the G-protein, which in stimulates membrane-bound adenylate cyclase. Activation of adenylate cyclase catalyses the conversion of ATP to cAMP. Upon up-regulation of cAMP, activation of protein kinase A occurs, resulting in protein phosphorylation and activation of the cAMP responsive element (Segaloff and Ascoli, 1993) (Figure 1.6).

Activation of protein kinase also activates the mitogen protein kinase pathways and a Janus-kinase signaling pathway (Angelova et al., 2002). All endocrine functions involve DNA transcription; for example, the elicitation of progesterone production in corpus luteal cells involves the synthesis of a cholesterol side-chain cleavage enzyme and fetal tissue growth involves protein synthesis. A parallel mechanism enhances the synthesis of an hCG/LH receptor binding protein. This activates exo- and endonucleases and leads to the destruction of receptor mRNA. This mechanism limits receptor expression, effectively down-regulating the receptor (Angelova et al., 2000) (Figure 1.6).

**Figure 1.6:** Activation of hCG/LH receptor, G protein and cAMP, protein kinase expression, and production of hCG/LH receptor binding protein (LHRBP). Synthesis of LHRBP activates exo- and endonucleases which destroy receptor mRNA, limiting expression and down regulating the receptor.

Modified from Cole, Reproductive Biology and Endocrinology 2010
Cancer vaccines

Cancer is a leading cause of death worldwide, accounting for 7.6 million deaths (around 13% of all deaths). The main types of cancer are: lung (1.4 million deaths), stomach (740 000 deaths), liver (700 000 deaths), colorectal (610 000 deaths), breast (460 000 deaths). Cancer is a major public health problem worldwide, requiring new strategies and treatment. In this context, immunotherapy has always been an attractive and potentially efficient treatment for cancer patients. Tumor immunotherapy can generally be classified as (i) passive, consisting of administration of cells or antibodies ex vivo, and (ii) active, represented by vaccines, aimed at eliciting a specific immune response against tumor-specific antigens (TSAs) and tumor-associated antigens (TAAs). Several types of cancer vaccines are now being developed, with a few reaching late stages of clinical evaluation (Figure 1.7).

Figure 1.7: Approaches to antitumor vaccination. (A) Irradiated tumor cells transduced with a viral gene transfer vector encoding a cytokine such as GM-CSF attract APCs (DCs) that acquire, process, and present tumor-associated antigens (TAAs) encoded by the vector in the context of MHC. (B) DCs can be directly loaded by incubation with tumor protein lysates or peptides with sequences based on expressed tumor antigens, or by viral gene transfer vectors.
expressing TAAs. (C) TAAs can be locally supplied to DCs by the direct injection of peptides, viral gene expression vectors, or naked DNA expression plasmids. DCs migrate to secondary lymphoid tissues where they present the antigen epitopes to T cells to generate an antitumor cytolytic T cell response.

**Tumor cell vaccines**

Tumor cell vaccines are made from actual cancer cells that have been removed during surgery. The cells are treated, usually with radiation, so they cannot form more tumors. The cells are then injected into the patient. The immune system recognizes antigens on these cells, then attacks any other cells with these antigens that are still in the body. There are two kinds of tumor cell vaccines: *autologous* and *allogeneic*.

*Autologous tumor cell vaccines:* *Autologous* means "coming from the self." An autologous tumor cell vaccine is made from killed tumor cells, taken from the same person in whom they will later be used.

*Allogeneic tumor cell vaccines:* *Allogeneic* means "coming from another." These vaccines use cells of a particular cancer type that originally came from someone other than the patient being treated. Some allogeneic tumor vaccines use a mixture of cells that were removed from several patients. The cells are treated and are usually injected along with one or more adjuvant substances to stimulate the immune system.

A number of genetically modified autologous or allogeneic tumor cell vaccines have been tested in clinical trials (Simons et al., 1999; Soiffer et al., 1998; Jaffee et al., 2001 and Salgia et al., 2003). Studies in patients with advanced prostate cancer (Simons et al., 1999) and metastatic malignant melanoma (Soiffer et al., 1998) used irradiated autologous tumor cells transduced with a retroviral vector expressing GM-CSF, resulting in one partial response in 21 melanoma patients, although extensive inflammatory infiltrate with necrosis and fibrosis of tumor was seen in biopsies from 11 of 16 melanoma patients.

**Antigen vaccines**

Antigen vaccines boost the immune system by using only one antigen, rather than whole tumor cells that contain many thousands of antigens. The antigens are usually proteins or peptides. Antigen vaccines may be specific for a certain type of cancer, but they are not made for a unique patient like autologous cell vaccines are. Some
antigens cause an immune response only in patients with a certain kind of cancer, while others produce immune reactions to more than one kind of cancer. Combinations vaccines can combine multiple antigens in an effort to induce stronger immune responses.

**Recombinant viral vectors**
These vaccines use special delivery systems (or vectors) to increase efficacy. Examples include vector-based antigen vaccines and vector-based DNA vaccines. A number of trials utilizing recombinant viruses expressing tumor antigens such as carcinoembryonic antigen (CEA) and prostate specific antigen (PSA) have been reported or are in progress; some of these constructs incorporate immunostimulatory molecules or cytokines (Marshall et al., 2000; Zhu et al., 2000; Hodge et al., 1999; Oh et al., 2003).

**Dendritic cell vaccines**
Dendritic cells (DCs), considered the most effective antigen-presenting cells (APCs), help initiate immune responses. As components of cancer vaccines, they present tumor antigens to T cells, facilitating the generation of anti-tumor cytotoxic T cell responses.

DC vaccines are autologous and must be made individually for each patient. DCs that present cancer antigens on their surface are able to trigger the patient’s immune system to recognize and destroy cancer cells that express the same antigens.

Clinical trials of DC vaccines have depended on development of techniques for obtaining large numbers of clinical grade human DCs (Caux et al., 1992; Sallusto and Lanzavecchia, 1994). Currently, two general approaches are used: (a) purification of immature DCs precursors from peripheral blood (Fong and Engleman., 2000); and (b) ex-vivo differentiation of DCs from CD34+ hematopoietic progenitor cells (Banchereau et al., 2001; Mackensen et al., 2000) or peripheral blood monocytes (Fong and Engleman., 2000), commonly by culture of monocytes with GM-CSF and IL-4 (Figure 1.8).
Immunization with mutant p53-peptide–pulsed DCs inhibited the growth of established tumors in the mice (Nair et al., 1997). Among 16 patients with metastatic melanoma receiving peptide- or tumor lysate-pulsed DCs injected directly into lymph nodes, 11 developed delayed-type hypersensitivity responses to peptide-pulsed DCs (Nestle et al., 1998). Among 11 melanoma patients receiving monocyte-derived DCs pulsed with HLA-A1–restricted MAGE-3 melanoma peptide, 8 developed a CTL response, and tumor regressions were observed (Thurner et al., 1999). Thus, the use of DCs as vehicles for therapeutic cancer vaccination holds promise.

![Figure 1.8: Generation of antitumor DC vaccines from peripheral blood monocytes. Monocytes are cultured with GM-CSF and IL-4 to produce DCs, which are then matured with CD40 ligand (CD40L) or other agents, pulsed with peptide or tumor lysate, or transduced with an expression vector and then injected into the patient as an autologous DC vaccine to induce a T cell immune response against the tumor.]

**DNA vaccines**

In such strategies, cells can be injected with bits of DNA that code for protein antigens. This DNA might be taken up by cells, resulting in the synthesis of target proteins. Intramuscular injections of naked DNA expression plasmids have been shown to generate immune responses (Tang et al, 1992; Ulmer et al., 1993). Such DNA vaccines introduce tumor antigen genes into DCs for endogenous processing and presentation to CTLs in draining lymph nodes or into other cells for cross-presentation by DCs, without the need for a viral vector.
Ectopic expression of hCG by non-gestational tumors was observed way back in 1904. In the late 1970s, common epithelial cancers were demonstrated to synthesize the hormone. Since the 1980s, reports describing the ectopic expression of hCG have grown (Iles and Butler, 1998).

**hCG in tumors**

**Germ cell tumors**

Germ cell tumors are a major type of gonadal neoplasms. Germ cell tumors comprise more than 90% of the tumors arising in the testis and 20% of those in the ovary. While 99% of testicular germ cell tumors are malignant, more than 90% of the ovarian tumors are benign teratomas. Thus, malignant germ cell tumors are 6–10 times more common in the testis than in the ovaries and gonadal tumors are also much more common than extragonadal tumors (Talerman, 1985 and Mayordoma et al., 1994). Female germ cell tumors, (dysgerminomas) are followed by endodermal sinus tumors, immature teratomas, mixed germ cell tumors, and embryonal carcinomas in terms of incidence (Zalel et al., 1996). Immature teratomas and yolk sac tumors are common during early childhood while dysgerminomas and mixed tumors are common in girls and young adults (Mayordoma, et al., 1994; Harms and Janig, 1986). Dysgerminomas often express hCG and may cause precocious puberty in young girls (Kurman and Norris, 1976; Roger et al., 1984). Other germ cell tumors, especially embryonal carcinomas, also express hCG. Yolk sac tumors regularly produce alpha-fetoprotein and only rarely hCG (Bosl and Motzer, 1997; Stenman and Alftan, 2002).

Testicular cancers are of two main types, seminomas and non-seminomatous germ cell tumors (NSGCT). More than 90% of NSGCTs contain a mixture of various histological types, that is, embryonal carcinoma, choriocarcinoma, teratomas, and yolk sac tumor, also-called endodermal sinus tumor (Bosl and Motzer, 1997; Mostofi et al., 1988). Tissue expression of hCG is detected in syncytiotrophoblasts of NSGCTs and in syncytiotrophoblastic components found in about 30% of seminomas (Mostofi et al., 1988). While hCG may be expressed by both seminomas and NSGCTs, high serum concentrations (>300–1000 IU/l) occur nearly exclusively in NSGCT (Ruther et al., 1994). Elevated serum concentrations of hCG occur in 40–50% of patients with NSGCT and 15–20% of those with seminomas (Mann et al.,
In introduction

1993). In addition, 20–40% of seminoma patients have elevated serum levels of hyperglycosylated hCG (hCG-H) alone (Mann et al., 1993; Saller et al., 1990). In a recent study, hCG-H was found to be expressed in 83% and hCG in 50% of the seminomas. In non-seminomatous tumors, incidence rates of the two forms were 72% and 82% respectively (Hoshi et al., 2000). Assays recognizing both hCG and hCG-H have been recommended for monitoring testicular cancers (Saller et al., 1990). However, because of the higher reference limit for hCG (16 pmol/l) compared with hCG-H (2 pmol/l), tumors producing only hCG-H will be detected later by an assay measuring hCG and hCG-H together than by a specific hCG-H assay.

**hCG in nontrophoblastic cancer**

The presence of hCG is observed in many nontrophoblastic tumors. The first description of a gonadotropin-producing nontrophoblastic tumor was a hepatoblastoma; presence of the hormone caused precocious puberty in a 2-year-old boy (Behrendt, 1930). This is a very rare condition with only 25 cases having been reported by 1985 (Nakagawara et al., 1985); of all cases of precocious puberty, only one-fifth of those in boys and a few percent in girls are caused by hCG-producing germ cell tumors (Roger et al., 1984). Occasional primary trophoblastic cancers have been observed in the lung (Hattori et al., 1979; Ikura et al., 2000 and Tsai et al., 2002) esophagus, stomach small bowel, colon, liver, breast, kidney, prostate and urinary bladder (Campo et al., 1989). In most studies on nontrophoblastic tumors, assays measuring both hCG and hCG-H have been used to measure hCG-immunoreactivity in serum. The overall incidence of elevated levels in various nontrophoblastic tumors (except testicular tumors) was 20% (Hussa, 1987). It is observed that the elevated values were mostly caused by hCG-H, but some patients also have slightly elevated hCG levels (Alfthan et al., 1992). Expression of hCG immunoreactivity at the tissue level has been demonstrated by radioimmunoassay of tissue extracts and by immunohistochemistry (Braunstein, 1975; Kurman and Norris, 1976). At the mRNA level, expression of hCGβ genes can be demonstrated by RT-PCR and sequencing both in normal and malignant tissues (Lazar et al., 1995). Specific assays for hCG subunits and ectopic expression were already described 30 years ago (Weintraub and Rosen, 1973 and Rosen and Weintraub, 1974), but they have not been widely used.
**Bladder cancer**

Among nontrophoblastic cancers, hCG immunoreactivity has been most extensively studied in transitional cell carcinoma (TCC) of the bladder and urinary tract. Expression has been detected by immunohistochemistry, and elevated serum levels have been found especially in patients with poorly differentiated tumors (Burry et al., 1986; Wurzel et al., 1987 and Martin et al., 1989). Depending on the type of patients studied, as well as the assay and cut-off used, the frequency of elevated levels ranges from 10% to 75% and rare cases of trophoblastic cancer in the bladder have been reported (reviewed in Iles and Chard, 1991). Bladder cancer cell lines often express hCG-H, but hCG expression has been observed both in malignant and “normal” urothelial cells (Iles and Chard, 1989). Expression of hCG immunoreactivity has been found to correlate with adverse prognosis (Iles et al., 1996).

**Renal cancer**

hCG expression in renal cancer was first detected by radioimmunoassay of concentrated urine (Fukutani et al., 1983) and in tissue by immunohistochemistry (Kuida et al., 1988). Studies with highly sensitive assays have shown that 23% of patients have elevated serum levels of hCG. The expression is not associated with tumor stage and grade, and an elevated serum level is an independent prognostic factor (Hotakainen et al., 2002). However, expression of hCG-H in tumor tissue as detected by immunohistochemistry and RT-PCR is not associated with prognosis (Hotakainen et al., 2003). Transitional cell carcinomas of the upper urinary tract also express hCG (Shah, 1987).

**Prostate cancer**

Increased hCG immunoreactivity has been observed in the urine of a few prostate cancer patients (Papapetrou et al., 1980; Fukutani et al., 1983 and Shah, 1987). The immunoreactive material in urine was found to consist of a low molecular form of hCG (Papapetrou et al., 1980), that is hCGhcf. In an early study, hCG was detected in serum of one of 16 prostate cancer patients (Broder et al., 1977). Expression of hCG has been observed by immunohistochemistry in less than 10% of prostatic adenocarcinomas, but by RT-PCR hCGβ mRNA has been detected both in normal and malignant prostatic tissue (Purnell et al., 1984 and Span, 2002).
Introduction

**Gastrointestinal cancers**

hCG immunoreactivity may occur in all cancers of the gastro-intestinal (GI) system. The overall incidence of elevated hCG plus hCG-H levels in serum as measured by radioimmunoassay is 21% (Hussa, 1987). The frequency of elevated values is higher in biliary (60%), pancreatic (46%), and gastric cancer (40%) (Alfthan et al., 1992 and Louhimo et al., 2002), and least frequent in liver (20%) (Braunstein et al., 1973) and colorectal cancer (15%) (Carplen et al., 1996 and Lundin et al., 2001). Its expression in hepatocellular cancer is rare (Zseli et al., 1984 and Louhimo et al., 2001), but elevated serum levels may occur in advanced disease (Marcillac et al., 1992 and Louhimo et al., 2002). Tissue expression of hCG-H has been detected with monoclonal antibodies in all GI cancers studied. The frequency of positive tumors is highest in gastric and pancreatic cancers and in cholangiocarcinomas (Webb et al., 1995 and Louhimo et al., 2001). Tissue expression or elevated serum levels of hCG-H have been found to be associated with adverse prognosis in most studies (Carplen et al., 1996; Lundin et al., 2001 and Louhimo et al., 2002).

**Neuroendocrine tumors**

Elevated levels of hCG subunits (in particular, hCGα) are frequently observed in patients with neuroendocrine tumors (Kahn et al., 1977 and Oberg et al., 1981). Carcinoid tumors most often express hCGα, while other neuroendocrine tumors may also express hCG-H (Oberg et al., 1981 and Grossman et al., 1994). Non-functioning pituitary tumors and somatotroph adenomas are often associated with elevated serum levels of hCGα (Ridgway et al., 1981 and Oppenheim et al., 1990) and tissue expression has been demonstrated in craniopharyngeomas (Tachibana et al., 1994).

**Lung cancer**

hCG immunoreactivity has frequently been observed in cell lines derived from non-small cell lung cancer, and less often from small cell lung cancer (Bepler et al., 1991). Tissue expression has been observed in 30–80% of the tumors by immunohistochemistry (Kuida et al., 1988; Wilson, 1981; Kimura and Ghandur-Mnaymen, 1985; Skrabanek et al., 1979 and Slowkowska et al., 1998). In serum, elevated Hcg immunoreactivity has been detected in 6% of various lung cancer patient (Burt et al., 1978) and Hcg-H levels above 5 IU/l in 12-14% of small cells. Lung cancers expression was associated with short survival (Szturmowicz et al., 1995)
**Introduction**

**Breast cancer**

Tissue expression of hCG-H has been detected to a variable extent in breast cancers by immuno-histochemistry (Kuida et al., 1988; Lee et al., 1985 and Agnantis et al., 1992). Biologically active hCG has been detected at concentrations of about 60 IU/l in breast fluid, indicating local production of the intact hormone in the breast (Abney et al., 1988). hCGβ mRNA level can be detected both in normal and malignant tissue by RT-PCR, and increased expression of the type 2 genes has been found to be a strong indicator of adverse prognosis (Bieche et al., 1998 and Span et al., 2003). Between 10% and 50% of patients with breast cancer have been found to have increased hCG immunoreactivity in serum (Braunstein et al., 1973; Tormey et al., 1977; Castro et al., 1979 and Caffier and Brandau, 1983).

**Head and neck cancer**

hCG immunoreactivity was observed in 64% of oral tumors by immuno-histochemistry, but only 0.5–5% of the cells were positive; staining was strongest in poorly differentiated tumors (Bhalang et al., 1999). Elevated serum levels of hCG-H have been observed in 14% of 59 patients with head and neck cancer before therapy; these patients experienced a shorter recurrence-free survival. In a Cox multivariate model, hCG-H levels and tumor stage were the only independent prognostic factors. In the same patients, the antigen showed no correlation with recurrence-free survival (Hedstrom et al., 1999).

**Hematological cancers**

Expression of hCG has been described in fresh and cultured lymphoma cells (Senba et al., 1991; Moller, 1996; Fraternali-Orcioni et al., 1999; Lazar et al., 1995 and Moller, 1996). hCG immunoreactivity has been observed in serum from several lymphoma patients (Braunstein et al., 1973 and Moller, 1996). Because expression of hCG-H genes can be detected in cultured lymphocytes from healthy subjects (Hotakainen et al., 2000), expression in haematological malignancies may simply constitute an up-modulation.
**Cell line culture**

COLO 205 (human colorectal cancer), ChaGo (human lung cancer), JEG-3 (human choriocarcinoma), OVCAR-3 (human ovarian cancer), CCl-253 (human colorectal adenocarcinoma), LLC (LL/2) (murine lung cancer) and MOLT-4 (human acute lymphoblastic leukaemia) were obtained from American Type Culture Collection (ATCC). Cell cultures were maintained at 37°C in 5% CO2 in a humidified incubator. Human cell lines were cultured in RPMI (GIBCO-BRL) supplemented with HEPES (2.36 g/l, Sigma) and NaHCO3 (2 g/l). Murine cell lines were cultured in DMEM (GIBCO-BRL) supplemented with HEPES (2.36 g/l, Sigma). Media were additionally supplemented with 10% Fetal Calf Serum (FCS, Biological Industries) and an antibiotic-antimycotic cocktail (GIBCO).

**mRNA expression of alpha and beta hCG**

**RNA Isolation**

All plastic ware was RNase free. Total RNA was isolated using a total RNA isolation kit (Intron). Briefly, 1 ml Lysis Buffer was added to 1x10⁶ cells. The mixture was vortexed at room temperature for 1 min then 200 µl chloroform was added, followed by additional mixing. A centrifugation was carried out at 18000 g at 4°C for 10 min. The upper layer was transferred to a fresh micro-centrifuge tube and 400 µl of Binding Buffer added, after mixing by gentle inversion, an incubation was carried out at RT for 1 min. The solution was “loaded” onto the spin column, followed by centrifugation 1 min at 18000 g. Wash Buffer A was added to the column, followed by similar centrifugation; Wash Buffer B was then added and the spin repeated. Elution Buffer (50 µl) was then added, after placing a fresh micro centrifuge collection tube below the column. After an incubation at RT for 3 min, a centrifugation was carried out at 18000 g for 1 min; RNA in the collection tube was either stored at -20°C or used immediately for further experiments.

**Generation of cDNA**

To 12 µl of RNA, 15 µl of DEPC treated water and 2 µl of oligo dT primers (Promega) were added. The mix was incubated for 10 mins at 70°C and then instantaneously chilled for 10 min on ice. 1.5 µl RNase inhibitor, 5X reverse transcriptase buffer, 2.5 µl dNTP mix (10 mM), 5 µl DTT (100 mM) and 2 µl AMV
RT (Promega) were then added. An incubation was carried out at 45°C for 1 hr followed by 70°C for 5 mins. cDNA was either stored at -20°C or used immediately for further experiments.

**Polymerase Chain Reaction**

All reagents were obtained from Promega. Primer sequences are given below:

Alpha hCG:  
Forward 5’-CTAGCTAGCATGGATTACTACAGAAAA- 3’  
Reverse 5’ -CGCGGATCCAGATTTGTGATAATAACA- 3’

Beta hCG:  
Forward 5’-CTAGCTAGCATGCTAAAGGGCTGCTG- 3’  
Reverse 5’-GCGATCGTGTCGCGCGGAGCAG- 3’

For a total reaction volume of 25 µl:

- c-DNA 2.5 µl
- Forward primer 2.0 µl
- Reverse primer 2.0 µl
- dNTP (10 mM) 0.5 µl
- Buffer 10X 2.5 µl
- MgCl₂ (50 mM) 1.0 µl
- Enzyme Taq polymerase 1.0 µl
- Nuclease-free H₂O 13.5 µl

The following PCR conditions were used:

- 94°C 5 min (Hot Start)
- 94°C 1 min Denaturation
- 55°C 1 min Annealing
- 72°C 1 min Extension
- 72°C 10 min Final Extension

After completion of the run, samples were stored at -20°C or used immediately for further experiments.
**Materials and methods**

**Expression of hCG and its role in tumor growth**

**Electrophoresis and gel elution**

Electrophoresis was carried out on a 1.2% agarose gel at 80V and the PCR product was visualized on a transilluminator. The band of interest were excised from the gel and transferred to a pre-weighed fresh micro centrifuge tube. The tube was weighed again to calculate the weight of the agarose slice and the band was eluted using a gel extraction kit (Qiagen). Briefly, 5 volumes (w/v) of the Buffer QG were added to the agarose slice and the mixture was incubated at 60°C for 15 minutes. The solution was then transferred to the column and centrifuged at 12,000 g for 1 minute at room temperature. The flow-through was discarded and 500 µl of Buffer QG was added to the column a centrifugation carried out at 12,000 g for 1 minute. 700 µl of Buffer PE was then added to the column and a centrifugation carried out at 12,000 g for 1 minute at room temperature. The flow through was discarded and columns were centrifuged at 12,000 g for 2 minutes at room temperature. To elute the DNA, 30µl of RNase-DNAse free water was added to the column and incubated at room temperature for 2 minutes followed by centrifugation at 12,000 g for 2 minutes at room temperature. The eluted DNA was stored at -20°C.

**Ligation**

The purified PCR product was ligated to PGMT-Ez vector (Promega). Following reaction was set up:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR amplified DNA</td>
<td>5.5 µl</td>
</tr>
<tr>
<td>Buffer 2X</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>Vector</td>
<td>1 µl</td>
</tr>
<tr>
<td>Enzyme</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

The mix was incubated at 16°C for 16 hrs.

**Transformation**

6 µl of the ligation mixture was added to competent E. coli DH5 alpha cells and an incubation carried out for 30 min on ice. Then cells were exposed to heat shock at 42°C for 90 sec and immediately transferred to ice for 5 min. 1 ml Luria Broth (Hi-media) was then added and an incubation carried out at 37°C for 1 hr with constant shaking. Cells were then centrifuged at 6800 g for 5 min; the supernatant was discarded and cells were dispensed onto LB agar plates containing IPTG (Hi-media),
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X-gal (Hi-media) and ampicillin. Plates were incubated at 37°C for 12-16 hrs.

**Plasmid purification**

White colonies were “picked” and inoculated into 3 ml of LB media containing ampicillin. Cultures were incubated at 37°C and then centrifuged at 5200 g for 20 min. Plasmid DNA was isolated using a plasmid isolation kit (Qiagen). 250 µl of Buffer P1 was added to the cell pellet and the mixture vortexed. 250 µl of Buffer P2 was then added and the mixture incubated for 2 min at room temperature. 300 µl of Buffer P3 was then added and tubes were incubated on ice for 15 min. A centrifugation was carried out at 15000 x g for 30 minutes at 4°C. To the supernatant, 560 µl of isopropanol was added and an incubation carried out at room temperature for 1 hour. Tubes were then centrifuged at 18000 g for 1 hour at 4°C. The supernatant was discarded and the pellet was “washed” once with 1 ml of 70% ethanol by repeated centrifugation. The resulting pellet was air-dried, resuspended in 50 µl of RNase-DNase free water and stored at -20°C.

**Restriction digestion**

The following reaction was set up for restriction digestion to verify the integrity of the insert:

- Plasmid DNA: 600 ng (2.0 µl)
- Eco R1 Buffer: 1.0 µl (1X)
- BSA: 1.0 µl (1X)
- Eco R1: 0.5 µl (5 units)
- H₂O: 5.5 µl (To a final volume of 10µl)

Tubes were incubated at 37°C for 16 hrs, following which agarose gel electrophoresis was carried out as before. After verifying release of an insert of appropriate size, plasmid DNA was sequenced using M13 primers either at a commercial facility or at the in-house sequencing facility at NII. Analysis was done using NCBI blast ([www.ncbi.nlm.nih.gov/nuccore/NM_000737.3](http://www.ncbi.nlm.nih.gov/nuccore/NM_000737.3)) ([www.ncbi.nlm.nih.gov/nuccore/NM_000735.3](http://www.ncbi.nlm.nih.gov/nuccore/NM_000735.3))

**Immunofluorescence Microscopy**

The reactivity of anti-hCG antibodies towards tumor cells was assessed by immunofl-
-uorescence microscopy. 5 x 10^5 cells were seeded into each well of a 24-well plate and incubation carried out at 37°C in a 5% CO_2 incubator for 16 hrs. The cells were “washed” with FACS Buffer (Appendix) by repeated centrifugation at 400 g for 5 mins at 4°C. To assess intra-cellular reactivity, cells were first permeabilized by incubation in Permeabilization Buffer (Appendix) for 90 secs and then “washed” three times with FACS buffer. Permeabilized and non-permeabilized cells were then incubated with Blocking Buffer (Appendix) for 1 hr at RT to block “non-specific” binding sites and “washed” once more with FACS Buffer. Cells were incubated with goat anti-hCG antiserum or normal goat serum (1:500 in FACS Buffer) for 2 hrs at RT. After three “washes” with FACS Buffer by repeated centrifugation, cells were incubated with an anti-goat FITC conjugate (Jackson ImmunoResearch; diluted 1:250 in FACS Buffer) for 2 hr at RT. Following subsequent “washes” with FACS buffer, digital images were acquired on a Nikon or Olympus fluorescence microscope.

**Flowcytometric analysis**

Surface and cytoplasmic reactivity on tumor cells of anti-hCG antibodies was also analysed by flow cytometry. 10^5 non-permeabilized or permeabilized cells were dispensed in each well of a 96-well round-bottom plate (Griener). Incubations with goat anti-hCG antibodies and the secondary conjugate were carried out as described above; for competition studies, the anti-hCG antibodies were first pre-incubated 1 µg hCG for 1 hr at 37°C. Data was acquired on a BD LSR Flowcytometer.

**Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot**

**Preparation of cell lysate**

COLO 205, ChaGo and LLC cells were harvested and “washed” with PBS three times by repeated centrifugation at 400 g for 5 mins at 4°C. TKM Buffer (Appendix) was added (150 µl per 5 x 10^6 cells) and an incubation carried out for 45 mins at 4°C. Cells were the snap-frozen by emersion in liquid nitrogen and then quick-thawed in four repeating cycles. The cellular material was centrifuged at 10500 g for 10 mins at 4°C. The supernatant, comprising the cellular lysate, was appropriately aliquotted and stored at -70°C.
**Protein estimation**

Protein concentration in the cell lysate was estimated by the BCA method (Pierce). BSA was used as standard and dilutions of lysate were made in TKM Buffer. Protein concentration were calculated by comparison with a standard curve subsequent to linear regression analysis.

**Electrophoresis**

Reagents and buffers for SDS-PAGE have been listed in the Appendix. Resolving and stacking gels were cast as per standard protocols. Sample Buffer was added to the samples and the mixture boiled for 10 min. Samples was then centrifuged at maximum speed in a micro-centrifuge to precipitate any debris. The supernatant (save the last 10 μl) were “loaded” into well on the gel and electrophoresis carried out at 60V. Once the samples “entered” the resolving gel, the voltage was increased to 80V. After completion of the run, the gel was processed for Western blot.

**Western blot**

The gel was immersed in Transfer Buffer. Nitrocellulose membrane (mdi) was cut to size of gel and soaked in Transfer Buffer. The nitrocellulose membrane was then placed on the gel, taking care to exclude any air bubbles. A padding of filter paper (Whatmann) was provided on both sides. Along with supportive sponges, the assembly was loaded into the transfer cassette which was then inserted into the transfer chamber, making sure the gel faced the anode. The chamber was then filled with Transfer Buffer. Transfer of moieties from the gel to the nitrocellulose membrane occurred upon application of current (70 mA for 16-20 hrs or 250 mA for 2 hrs) at 4 °C. Protein moieties transferred to the nitrocellulose membrane were visualized by brief incubation with the protein-staining dye Ponceau S (Sigma). The membrane was then washed and “de-stained” with PBS. Non-specific sites on the nitrocellulose membrane were “blocked” by incubation with TBS containing 3% BSA for 2 hrs at RT. The membrane was then “washed” six times with TBS containing 0.05% Tween-20 (TBST); washes involved 10 min incubations at RT with gentle rocking. Primary antibody (goat anti-hCG antiserum or normal serum diluted 1:500 in TBS containing 0.05% Tween-20 and 1% BSA) was added to membrane strips and an incubation carried out for 2 hrs at RT; for competition studies, antiserum was first pre-incubated
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with 1 µg hCG for 1 hr at 37°C. Strips were “washed” extensively with TBST to remove unbound antibodies. A rabbit anti-goat HRP conjugate (diluted 1: 8000 in TBS containing 0.05% Tween-20 and 1% BSA) was added to the strips and incubation carried out for 1 hr at RT. After further washes, enzyme activity was visualized by chemiluminescence (ECL, Biological Industries). Briefly, equal volumes of the two supplied reagents were mixed and the solution incubated with nitrocellulose membrane for 3-5 mins in the dark. The reagent was then drained and membrane was exposed to X-ray film (Kodak) which was then developed.

Effect of hCG and anti-hCG antibodies on tumor cell viability in vitro

COLO 205, ChaGo, CCL-253 and LLC cells were re-suspended in serum-free medium (Bio Whittaker). 3 x 10^5 cells per well were dispensed in a 96-well plate and an incubation carried out for 16 hrs at 37°C in a 5% CO₂ incubator. hCG (1 µg), anti-hCG antiserum (1:100), non-immune serum (1:100), hCG + anti-hCG antiserum or hCG + non-immune serum were individually dispensed and an incubation carried out for 24 hrs under standard culture conditions. CCL-253 cells were incubated with serum-free medium containing anti-hCG antiserum or non-immune serum with or without 14% complement. Cells were then “washed” with PBS by three centrifugations at 400g for 5 min at 4°C. 80 µl of a 5 mg/ml solution of an MTT reagent (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide; Sigma) was then added, followed by an incubation for 5 hrs. 50 µl of the Stop Solution (50% DMSO in 20% SDS) was dispensed and incubation carried out for 1 hr at RT. Cell proliferation/viability was determined colorimetrically by measuring optical density at 550 nm. A Trypan Blue dye exclusion assay was employed as an additional assessment of cell death.

Effect of hCG on tumor cell growth in vivo

The effects of hCG on tumor growth were assessed in vivo. Female C57BL/6 inbred mice (6-8 weeks old) were subcutaneously implanted with 10^4 syngeneic LLC cancer cells. Test animals were intra-peritoneally administered either 100 ng or 1 µg hCG twice a week for the duration of the experiment (60 days); control animals received
PBS. Tumor volumes were measured periodically using digital vernier calipers and tumor volumes estimated using the formula $\frac{4}{3}\pi r^3$. 
Detection of αhCG and βhCG transcripts in tumor cell lines

PCR amplification of mRNA transcripts for α and β subunits of hCG was carried out using specific primers, as described in the Materials and Methods section. For both COLO 205 and ChaGo cells, PCR products of \( \approx 350 \) bp for alpha hCG (Figure 1A) and \( \approx 500 \) bp for beta hCG (Figure 1B) were obtained, indicating active transcription.

Reactivity of anti-hCG antibodies towards tumor cells

Cell surface and intracellular reactivity of anti-hCG antibodies was analyzed by indirect immunofluorescence microscopy. COLO 205, ChaGo, LLC, OVCAR-3, JEG-3, CCL-253 and MOLT-4 cells demonstrated surface and cytoplasmic reactivity; no such recognition by antibodies in normal serum (used as a negative control) was observed (Fig 2A-G). In contrast, anti-hCG antibodies were non-reactive towards Jurkat cells (Figure 2H).

Flow cytometric analysis on tumor cells confirmed the surface and intracellular reactivity of anti-hCG antibodies. Competition assays using hCG provided further proof of specificity; pre-incubation of the anti-hCG antibodies with hCG induced a significant decrease in both surface and cytoplasmic reactivity (Figure 3).

Western blot analysis on whole cell lysates revealed reactivity of anti-hCG antibodies towards several moieties. Pre-incubation of the antibodies with hCG resulted in a reduction of reactivity; decreases in band intensity were observed in many cases. Normal goat serum, employed as an isotype control, was non-reactive (Figure 4). These results suggest that many moieties demonstrating cross-reactive recognition of anti-hCG antibodies possibly exist on tumor cells.

The effect of hCG on the growth of tumor cells in vitro and in vivo

hCG was demonstrated to have growth-promoting effects on tumor cells; hCG enhanced cellular growth (or increased cellular viability) in a dose-dependent manner in vitro as measured by an MTT assay (Figure 5).

The growth-promoting effects of hCG were confirmed in vivo. C57BL/6 mice were subcutaneously implanted with \( 10^4 \) LLC cells. Tumors in mice administered 1 µg hCG
(per injection, three injections a week for 6 weeks) grew faster than in mice receiving 100 ng hCG or buffer (Figure 6A, B).

**The effect of anti-hCG antibodies on the growth of tumor cells in vitro**

The influence of anti-hCG antibodies on cellular viability was then assessed; antibodies significantly inhibited cellular viability/growth, whereas normal serum, employed as an isotype control, had no effect (Figure 7). The inhibitory effects of anti-hCG antibodies on COLO 205, ChaGo and LLC were dose-dependent; the viability of Jurkat cells, which were non-reactive to anti-hCG antibodies, remained unaffected in their presence (Figure 8). The addition of complement significantly enhanced the inhibitory effects of anti-hCG antiserum on CCL-253 (Figure 9).
Figure 1: Detection of (A) αhCG and (B) βhCG mRNA in tumor cells by reverse transcriptase-PCR. Lane 1: COLO 205 cells; Lane 2: ChaGO cells; M: 100bp marker ladder
Figure 2A: Reactivity of (i, iii) anti-hCG antiserum or (v, vii) normal serum towards (i, v) non-permeabilized and (iii, vii) permeabilized COLO 205 cells by indirect immunofluorescence. ii, iv, vi and viii represent corresponding phase contrast images.
Figure 2B: Reactivity of (i, iii) anti-hCG antiserum or (v, vii) normal serum towards (i, v) non-permeabilized and (iii, vii) permeabilized ChaGo cells by indirect immunofluorescence. ii, iv, vi and viii represent corresponding phase contrast images.
Figure 2C: Reactivity of (i, iii) anti-hCG antiserum or (v, vii) normal serum towards (i, v) non-permeabilized and (iii, vii) permeabilized LLC cells by indirect immunofluorescence. ii, iv, vi and viii represent corresponding phase contrast images.
Figure 2D: Reactivity of (i, iii) anti-hCG antiserum or (v, vii) normal serum towards (i, v) non-permeabilized and (iii, vii) permeabilized OVCAR 3 cells by indirect immunofluorescence. ii, iv, vi and viii represent corresponding phase contrast images.
Figure 2E: Reactivity of (i, iii) anti-hCG antiserum or (v, vii) normal serum towards (i, v) non-permeabilized and (iii, vii) permeabilized JEG3 cells by indirect Immunofluorescence. ii, iv, vi and viii represent corresponding phase contrast images.
Figure 2F: Reactivity of (i, iii) anti-hCG antiserum or (v, vii) normal serum towards (i, v) non-permeabilized and (iii, vii) permeabilized CCL-253 cells by indirect immunofluorescence. ii, iv, vi and viii represent corresponding phase contrast images.
Figure 2G: Reactivity of (i, iii) anti-hCG antiserum or (v, vii) normal serum towards (i, v) non-permeabilized and (iii, vii) permeabilized MOLT-4 cells by indirect immunofluorescence. ii, iv, vi and viii represent corresponding phase contrast images.
Figure 2H: Reactivity of anti-hCG antiserum on (i) non-permeabilized and (iii) permeabilized Jurkat cells by indirect immunofluorescence. ii, iv represent corresponding phase contrast images.
Figure 3: Flow cytometric analysis on (A-F) non-permeabilized and (A’-F’) permeabilized tumor cells. (A, A’) ChaGo; (B, B’) COLO 205; (C, C’) CCL-253; (D, D’) OVCAR; (E, E’) MOLT-4; (F, F’) LLC. Red profiles: Negative control; Black profiles: reactivity of anti-hCG antibodies; Green profiles: reactivity of anti-hCG antibodies after pre-incubation with hCG.
Figure 4: Western blot analysis of the reactivity of cellular lysates derived from (A) COLO 205 (B) ChaGO (C) LLC (D) JEG3 (E) OVCAR cells and (F) CCL-253. Lane 1: Anti-hCG antiserum; Lane 2: Anti-hCG +hCG; Lane 3: NGS; Lane 4: NGS + hCG.
Figure 5: Influence of hCG on cell viability as assessed in an MTT assay. (A) COLO 205, (B) ChaGO and (C) LLC cells. *p = 0.01 vs control cultures.
Figure 6: (A) Effect of hCG administration (at 100 ng or 1 μg per injection) on the growth tumors induced subsequent to the subcutaneous implantation of LLC cells in C57BL/6 mice. (B) Individual and mean tumor volumes at Day 47 in mice administered hCG or buffer. ***p < 0.001
Figure 7: Influence of anti-hCG antibodies (1:500) on cell viability as assessed by an MTT assay. (A) COLO 205, (B) ChaGO and (C) CCL-253 (D) LLC cells. *p < 0.05, **p < 0.01. NS: Normal Serum.
Figure 8: Influence of different dilutions of anti-hCG antiserum on cell viability as assessed by an MTT assay. (A) COLO 205, (B) ChaGO, (C) LLc and (D) Jurkat cells.
Figure 9: Influence of anti-hCG antibodies (1:500) with and without complement on viability of CCL-253 as assessed by an MTT assay. **p<0.01. *p<0.05, ns= not significant.


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