hCG was initially believed to be a pregnancy-specific hormone. More recent studies suggest that it is also secreted by a variety of tumors. Ectopic production of a bioactive hCG-like material by nongestational tumours was reported as early as 1946 (McFadzean, 1946). After the introduction of more specific and sensitive immunological assays for the beta-subunit of the hormone, many more tumours, particularly those derived from germ cells, have been recognised as hCG producers (Braunstein et al., 1973; Javadpour et al., 1978a, b). The highest incidence of ectopic expression of hCG by epithelial carcinomas is found in islet cell carcinomas (45%) followed by ovarian carcinoma (39%) (Braunstein, 1983). Detection of very low levels of hCG by epithelial tumours has also been demonstrated (Adejuwon et al., 1980; Braunstein, 1983).

In the present study, anti-hCG antibodies were assessed for reactivity towards cell lines of different lineages (human colon, human colorectal, human and murine lung, human ovarian, human leukemic, human choriocarcinoma) by immunofluorescence and flow cytometry. Such analysis demonstrated both cell surface and intracellular expression of antibody-reactive moieties. The specificity of antibody interaction was confirmed by competition studies using hCG, as well as by a demonstrated lack of cellular reactivity of antibodies in normal serum. Additionally, the presence of transcripts for both subunits of hCG was demonstrated in several tumor lineages by reverse transcriptase PCR.

Amongst the multiple roles hCG plays to ensure the success of pregnancy, it also promotes the growth of cytotrophoblast cells and differentiation of the placenta. hCG has also been shown to function in umbilical cord growth (Rao et al., 1993; Rao et al., 1995). hCG (along with its hyperglycosylated form) appears to be involved in multiple steps of placentation and fetal development (Goldsmith et al., 1983; Abdallah et al., 2004; Rao et al., 2010; Rao et al., 2001; Rao and Lei, 2007 and Rao et al., 1995; Rao et al., 1993; Wasowicz et al., 1999). In the present study, it was demonstrated that hCG works as a potent growth factor for tumor cells both in vivo and in vitro. Tumor cells incubated with hCG demonstrated dose-dependent growth, an effect significantly inhibited by anti-hCG antibodies. Further, tumors arising in C57BL/6 upon the implantation of LLC cells mice grew at a faster rate upon the
administration of hCG. Hence, the growth-promoting effects of hCG, previously documented for reproductive tissues, extends to tumor cells as well.

VEGF and IL-8 are considered key angiogenic factors associated with vascularisation in tumors. The first VEGF-targeted agent, the anti-VEGF monoclonal antibody bevacizumab (Avastin, Genentech), showed clinical benefit in patients with metastatic colorectal cancer (CRC) when combined with chemotherapy (Hurtwiz et al., 2004). A role for VEGF in abnormal DC differentiation was first demonstrated in vitro and addition of neutralizing VEGF antibodies resulted in normal maturation of DCs (Gabrilovich et al., 1996). VEGF can stimulate vasculogenesis in tumours by recruiting bone marrow-derived haematopoietic progenitor cells (HPCs) and endothelial progenitor cells (EPCs) (Bertolini et al., 2006 and Lyden et al., 2001). IL-8 expression is enhanced by vascular endothelial growth factor (VEGF)-A, and both are modulated by hypoxia (Rofstad and Halsor, 2002; Belperi et al., 2000). The chemotactic response of melanoma cells to IL-8 was mediated by CXCR1 (Ramjeesingh et al., 2003), and the mitogenic activity of IL-8 was mediated by both CXCR1 and CXCR2 in colon cancer (Li et al., 2001), but only by CXCR1 in monocytes (Browning et al., 2000). There is evidence of crosstalk between the IL-8 and epidermal growth factor (EGF) receptors (Venkatakrishnan et al., 2000). IL-8 has been established as a proangiogenic factor and also regulates tumorogenicity and metastasis in androgen-dependent cancers (Xie, 2001, Rofstad et al., 2000; Inoue et al., 2000).

hCG has been demonstrated to play a role in angiogenesis. Acting via hCG/LH receptors on uterine spiral arteries, hCG functions to promote angiogenesis and vasculogenesis in the uterine vasculature during pregnancy (Rao et al., 1993; Zygmunt et al., 2003), ensuring an adequate supply of blood to the invading placenta and optimal nutrition to the fetus (Berndt et al., 2009; Toth et al., 1994; Lei et al., 1992; Zygmunt et al., 2002; Herr et al., 2007; Zygmunt et al., 2003 and Toth et al., 2001). hCG is now considered a potent and significant angiogenic factor in pregnancy (Zygmunt et al, 2002). hCG was shown to stimulate angiogenesis in vivo and in vitro and also to up-modulate the secretion of VEGF by endothelial and epithelial cells (Berndt et al., 2006). hCG can up-modulate VEGF secretion during the implantation process (Licht et al., 2007). VEGF contribution in angiogenic activity may be due to
its ability to enhance survival by inducing expression of Bcl-2 (Nör et al., 1999). hCG has been previously shown to elicit the production of IL-8 by monocytes independently of the LH/HCG receptor (Kosaka et al., 2002). Of interest is the fact that heightened serum hCG levels correlate with vascularisation in testicular germ cell tumors (Oscar et al., 2009). In the current studies, it was observed that hCG up-regulated transcription and secretion of both VEGF and IL-8 in tumor cells, effects dampened by anti-hCG antibodies. In a scenario where autocrine up-modulation of these critical mediators can be postulated to occur in vivo, anti-hCG vaccination could offer significant clinical benefit.

The biochemical pathways by which hCG induces the generation of VEGF and IL-8 from tumor cells were then investigated, using specific inhibitors. The hCG/LH receptor is expressed in gonadal and in non-gonadal tissues of the female reproductive system (Lie et al., 1993), in the pregnant and non-pregnant uterus (Licht et al., 2003), fallopian tube, amnion, chorion, decidua (Toth et al., 1996), umbilical cord (Rao et al., 1993), and placenta. hCG interacts with LH/hCG G-protein-coupled receptor and activates the cAMP pathway (Ascoli et al., 2002). It has been demonstrated that LH/hCG induces VEGF transcription in granulosa cells (Christenson et al., 1997; Neulen et al., 1998) and in the rat ovary (Koos, 1995) and evidence suggested that cAMP/PKA pathway is involved in angio-stimulation by hCG (Berndt et al., 2002). Other investigators have shown that the PKC transduction pathway plays an important role in capillary formation and survival phases of angiogenesis (Krugar et al., 1998; Davis et al., 1993; Xia et al., 1996 and Ilan et al., 1993). The influence of hCG on the process of angiogenesis may occur directly via the hCG/LH receptor and downstream molecules (e.g. PKC), or indirectly by increasing the production of established angiogenic factors or other growth factors (e.g. EGF). In the current study, the MAPK and PKA pathways were found to be involved in the hCG-mediated up-modulation of VEGF and IL-8 in tumor cells.

Matrix metalloproteinases (MMPs) are a family of structurally related zinc- and calcium-dependent endopeptidases that can degrade extracellular matrix (ECM) components (Egeblad and Werb, 2002; Overall and Lopez-Otin, 2002). MMP-mediated proteolysis is involved in various physiological and pathological processes such as embryonic development, involution, wound healing, inflammatory diseases,
cancer invasion, and metastasis (Sternlicht and Werb, 2001). Among the MMPs, MMP-2 (gelatinase A), and MMP-9 (gelatinase B) are especially important in collagen degradation (Véronique et al., 2004). Growth factors such as VEGF that are sequestered in the extracellular matrix are activated by MMP-9-mediated proteolysis (Bergers et al., 2000). Based on the assumption that MMPs were responsible for metastasis, several orally active inhibitors of MMPs (MMPIs) have been developed, with some found effective in controlling cancer progression in animals (Zuckers et al., 2004). hCG prevents luteolysis by maintaining luteal blood flow, limiting tissue remodelling through a reduction of matrix metalloproteinase-2 (MMP-2) activation and macrophage influx (Duncan et al., 2000), and preventing apoptosis via an increase in the BclII/Bax ratio (Sugino et al., 2000). hCG also has a regulatory role on the MMP/TIMP system at the implantation site (Fluhr et al., 2008). The present study demonstrates that hCG up-modulates transcription and expression of MMP-2 and MMP-9 in tumor cells. These effects can be postulated to assist cancer cells invade and metastasize. Neutralization of such tumor-promoting effects by anti-hCG vaccination could aid in prolonging patient survival.

Previous reports suggest that hCG acts as a chemotactic signal, inducing the migration and invasion of trophoblast cells (Zygmunt et al., 1998b; Zygmunt et al, 2005). In the present study, hCG was observed to behave as a chemotactic signal for endothelial cells, while simultaneously enhancing their proliferation. Similar results have previously reported by other investigators, where hCG induced both endothelial cell proliferation and sprouting in vitro (Berndt et al., 2006 and Herr et al., 2007).

At picomolar concentrations, hCG has been previously demonstrated to function as a chemoattractant for neutrophils, monocytes and lymphocytes in vitro (Reinisch et al., 1994). This study found that hCG could induce the preferential migration of monocytes and T cells; the migration of B cells was not affected. The molecular mechanisms behind these intriguing results remain unclear at present. However, some down-stream consequences of such an effect can be postulated. An increase in monocyte numbers could possible translate into an increase in the secretion of pro-inflammatory, tumor-associated cytokines via the autocrine/paracrine mechanisms described in this study. It also remains to be determined whether hCG induces the differential migration of different sub-populations of T cells. Previous reports suggest
that hCG can preferentially attract Tregs towards the feto-maternal interface and may be important in mediating tolerance towards paternal antigens (Schumacher et al., 2009). Whether tumor-derived hCG can function similarly is clearly an issue of some significance, given the fact that the accumulation of Tregs in solid tumors is believed to contribute towards the suppression of anti-tumor immune responses and poor patient prognosis (Zhou et al., 2009).

Reports suggest that Tregs suppress anti-tumor responses in colorectal cancer (Clarks et al., 2006). Infiltrating Tregs can thus create a niche for enhanced tumor growth. CD4⁺FoxP3⁺ Tregs act as a dominant immune escape mechanism for gliomas (Oliver et al., 2007). Other FoxP3⁺ cells may also be important in this regard; recent studies demonstrate the existence of a sub-population of naturally-occurring macrophage regulatory cells in which expression of Foxp3 correlates with immune suppressive function (Manrique et al., 2011). However, FoxP3 has been most often studied as a master regulator in the development and function of regulatory T cells (Zhang and Zhao, 2007, Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003; Sakaguchi et al., 2008; Zheng and Rudensky, 2007). Transforming growth factor-β (TGF-β) and Interleukin-10 (IL-10) are two principal soluble immunosuppressive mediators secreted by Tregs cells. TGF-β is a multifunctional cytokine which regulates T cell growth and development (Wahl, 1994; Massague, 1996 and Massague et al, 2000). TGF-β induces the differentiation of CD4⁺CD25⁻ T cells into CD4⁺CD25⁺ Tregs which are then capable of suppressing T-cell cytotoxic activity and inhibiting antibody production (Moo-Young et al, 2009). IL-10 mediates potent anti-inflammatory and immunosuppressive effects (Saraiva and O’Garra, 2010).

Recent reports suggest that some tumor cells also express FoxP3 (Hinz et al, 2007 and Karanikas et al, 2008), thereby suggesting that such cells may actively contribute in the creation of an immunosuppressive milieu. In this study, it was determined that hCG up-modulates FoxP3 expression in some tumor cells; while increases were observed in COLO-205 (human colon cancer) and LLC (mouse lung cancer), no such effects were observed in ChaGo (human lung cancer) cells. Supernatants of tumor cells incubated with hCG contained enhanced concentrations of both TGF-β and IL-10; such supernatants induced the conversion of CD4⁺CD25⁻ cells into CD4⁺CD25⁺ T cells. Thus, hCG can mediate immune suppression in two significant ways: It can
Discussion

directly induce the generation of immunosuppressive cytokines as well as induce the generation of regulatory cells that can mediate suppression.

IDO is a cytosolic monomeric hemoprotein (MW 45,324 Da) that catalyses the first step in tryptophan catabolism by the kynurenine pathway (Terentis et al., 2002), creating a tryptophan-deficient microenvironment (Kudo and Boyed 2000, 2001). Cancer cells as well as APCs in the tumor, in the peritumoral stroma and in tumor-draining lymph nodes have been shown to express IDO (Mellor and Munn, 2004). In addition, the expression of IDO has been shown both in human tumour cell lines and in primary tumours (Uyttenhove et al., 2003; Taylor and Feng, 1991). An initial hypothesis considered that the capacity of IDO to deplete tryptophan in the medium could be an IFN-induced mechanism that inhibits tumour proliferation (Ozaki et al., 1988). More recently it has been suggested that tryptophan depletion (along with the effects of break-down products catabolism by the kynurenine pathway) may function to suppress the immune system. A similar mechanism possibly contributes towards the prevention of rejection of an allogeneic fetus (Mellor et al., 2002); IDO at the maternal-fetal interface creates a local microenvironment that precludes maternal T cell activation to fetal alloantigen (Munn et al., 1998). In the present study, tumor cells up-modulated CTLA4 upon incubation with hCG, possibly under the influence of FoxP3. Incubation of such cells with mature DCs cells elicited the generation of IDO. It is postulated this effect was due to the interaction of CTLA4 with co-stimulatory molecules CD80/CD86 on dendritic cells. This data reveals another potential pathway by which hCG can help create an immunosuppressive environment for tumor cells.

There exists a critical link between inflammation and cancer. Indeed, growing evidence appears to suggest that many malignancies are initiated by microbial infections (Kuper et al., 2000, Blaser et al., 1995, Scholl et al., 1994). In this present study, hCG was found to up-modulate the secretion of versican, a large proteoglycan. Versican is up-modulated in many cancers and plays a role in cell proliferation, apoptosis, motility, adhesion, invasion, angiogenesis and metastasis (Ricciardelli et al., 2009). Supernatants of tumor cells incubated with hCG induced the generation of the inflammatory cytokines IL-6 and TNF-α from monocytes, possible as a result of
the binding of versican with TLR-2 (Kim et al., 2009); heightened expression of both cytokines have been associated with tumorigenesis.

The principal findings of this study have been summarized in Figure A. The data suggests that hCG can mediate a variety of direct and indirect effects on tumor cells and on cells of the immune system. Several of these effects have been shown in independent studies to directly impact tumor growth and metastasis. In addition to having a positive influence on cellular growth, the molecule appears to influence cells and molecules involved in immune suppression, inflammation, angiogenesis and metastasis. Possibly via the induction of the transcription factor FoxP3, hCG induces the secretion of the immunosuppressive cytokines IL-10 and TGF-β, as well as mediates an up-modulation of cell surface CTLA4. The former event can potentially drive the differentiation of CD4⁺CD25⁻ cells into CD4⁺CD25⁺ Treg cells, which can further perpetuate immunosuppressive effects. Possibly as a consequence of CTLA4 interacting with CD80/CD86 on mature DCs, the DCs make IDO, an enzyme known to induce local immune suppression as a result of its tryptophan catabolising action. hCG also induces the secretion of the proteoglycan versican, which further induces the secretion of the inflammatory cytokines TNF-α and IL-6 from monocytes. hCG can up-modulate the secretion of VEGF and IL-8 from both cancer cells and endothelial cells, leading to neoangiogenesis and the proliferation of endothelial cells. Simultaneously, hCG also up-modulates the expression of MMPs (specially MMP2 and MMP9) in cancer cells. These dual processes can act in conjunction to induce invasion and metastasis.
Figure A: Summary of the main findings of this study