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ORIGINAL ARTICLE

Colon cancer cells produce immunoregulatory glucocorticoids

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Glucocorticoids (GC) have important anti-inflammatory and pro-apoptotic activities. Initially thought to be exclusively produced by the adrenal glands, there is now increasing evidence for extra-adrenal sources of GCs. We have previously shown that the intestinal epithelium produces immunoregulatory GCs and that intestinal steroidogenesis is regulated by the nuclear receptor liver receptor homolog-1 (LRH-1). As LRH-1 has been implicated in the development of colon cancer, we here investigated whether LRH-1 regulates GC synthesis in colorectal tumors and whether tumor-produced GCs suppress T-cell activation. Colorectal cancer cell lines and primary tumors were found to express steroidogenic enzymes and regulatory factors required for the *de novo* synthesis of cortisol. Both cell lines and primary tumors constitutively produced readily detectable levels of cortisol, as measured by radioimmunoassay, thin-layer chromatography and bioassay. Whereas overexpression of LRH-1 significantly increased the expression of steroidogenic enzymes and the synthesis of cortisol, downregulation or inhibition of LRH-1 effectively suppressed these processes, indicating an important role of LRH-1 in colorectal tumor GC synthesis. An immunoregulatory role of tumor-derived GCs could be further confirmed by demonstrating a suppression of T-cell activation. This study describes for the first time cortisol synthesis in a non-endocrine tumor in humans, and suggests that the synthesis of bioactive GCs in colon cancer cells may account as a novel mechanism of tumor immune escape.
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Introduction

Colorectal cancer (CRC) is one of the leading causes of death in the Western world. It develops through a series of genetic mutations in pluripotent stem cells in the intestinal epithelial crypts (Davies *et al*., 2005). Many cancer cells aberrantly overexpress cellular proteins or neo-antigens, which can be recognized by the immune system and provoke anti-tumor immune responses (van den Broek *et al*., 1996; Smyth *et al*., 2000; Shankaran *et al*., 2001). Also, CRC cells are often immunogenic and can stimulate the activation of tumor-specific lymphocytes (Camus *et al*., 2009). Therefore, anti-tumor immune responses may significantly contribute to tumor immune surveillance and limit tumor development and growth. Leukocytes frequently infiltrate neoplastic tissue indicating ongoing anti-tumoral immune responses, yet their specific role in tumor surveillance is unclear. Activation of tumor-infiltrating immune cells and the release of pro-inflammatory mediators may not only control tumor development but also, in the contrary, stimulate angiogenesis and thereby accelerate tumor growth. However, recent studies by Pages *et al*., 2005 and Galon *et al*., 2006 revealed that the number of tumor-infiltrating lymphocytes in CRC directly correlates with a better prognosis and longer survival of CRC patients. This finding indicates that anti-CRC immune responses are likely substantially contributing to tumor surveillance. Still, in most patients, the anti-tumor immune response seems to be unable to control CRC growth and metastasis formation in a long term. Immune escape mechanisms have been recognized as one of the hallmarks of cancer (Hanahan and Weinberg, 2000; Finn, 2008) and may significantly limit the efficacy of anti-tumor immune responses.

Glucocorticoids (GC) are lipid hormones that are critically involved in the regulation of stress responses, metabolism and immune homeostasis (Sapolsky *et al*., 2000). The adrenal glands are the major source of GC in the body. However, different reports indicate that GC synthesis is not restricted to the adrenal glands but may also occur at other sites of the body (Noti *et al*., 2009), for example, the thymus (Vacchio *et al*., 1994), the brain (MacKenzie *et al*., 2000; Davies and MacKenzie, 2003) and the vascular endothelium (Takeda *et al*., 1994). Recently, we have characterized the intestinal epithelium as a major source of immunoregulatory GC

(Cima *et al*., 2004; Mueller *et al*., 2006, 2007; Atanasov *et al*., 2008). In mice, intestinal GC synthesis is highly regulated and is induced upon activation of immune cells. In turn, intestinal GCs have been shown to exert paracrine activities and to contribute to the regulation of intestinal immune cell homeostasis. Thus, mice with defective intestinal GC synthesis show enhanced antigen-induced activation of intestinal T cells (Cima *et al*., 2004) and accelerated induction of experimental inflammatory bowel disease (Coste *et al*., 2007).

The synthesis of GC from cholesterol involves a series of enzymatic steps catalyzed by steroidogenic enzymes of the cytochrome P450 (CYP) and the hydroxysteroid dehydrogenase family (Miller, 2008). In the intestine, steroidogenesis is critically regulated by the nuclear receptor and transcription factor liver receptor homolog-1 (LRH-1, NR5A2; Mueller *et al*., 2006, 2007; Coste *et al*., 2007). Consequently, absence of LRH-1 results in strongly reduced intestinal GC synthesis (Mueller *et al*., 2006) and enhanced susceptibility to intestinal inflammatory processes (Coste *et al*., 2007). The function of LRH-1 in the intestinal crypts is, however, not limited to GC synthesis, but further extends to the control of cell cycle and tumorigenesis (Botrugno *et al*., 2004; Schoonjans *et al*., 2005). Accordingly, LRH-1 haplo-insufficient mice have reduced tumor formation in a mouse model for intestinal cancer (Schoonjans *et al*., 2005).

Here, we demonstrate that CRC cell lines and primary tumors express steroidogenic enzymes and produce bioactive GC. LRH-1 was found to be abundantly expressed in CRC cells and to critically regulate steroidogenesis in tumor cells. Finally, we provide evidence that tumor cell-produced GCs inhibit the activation of T lymphocytes. These findings indicate that CRC cells have a strong steroidogenic potential and that tumor-derived GC may contribute to tumor immune evasion.

Results

Colon cancer cell lines express steroidogenic factors transcription factor SF-1

GCs are produced from cholesterol via a series of enzymatic steps involving enzymes of the CYP and the hydroxysteroid dehydrogenase family. Most of these enzymes are transcriptionally regulated by tissue-specific nuclear receptors, such as LRH-1 in the intestine (Miller, 2008). We thus examined the expression of LRH-1 and different steroidogenic enzymes required for GC synthesis in various established CRC cell lines by reverse transcription–PCR. Figures 1a–d illustrate that the steroidogenic enzymes CYP11A1 and CYP11B1 were expressed at relatively high levels in HT29, Caco2 and LS174T cells, whereas CY17 was not detected in LS174T cells. Similarly, LRH-1 was expressed at high levels in Caco2 and HT29 cells, but was absent in LS174T cells.

LRH-1 overexpression is sufficient to induce steroidogenic enzyme expression in CRC cell lines

Regulation of steroid synthesis in the adrenal glands differs substantially from that in the intestine

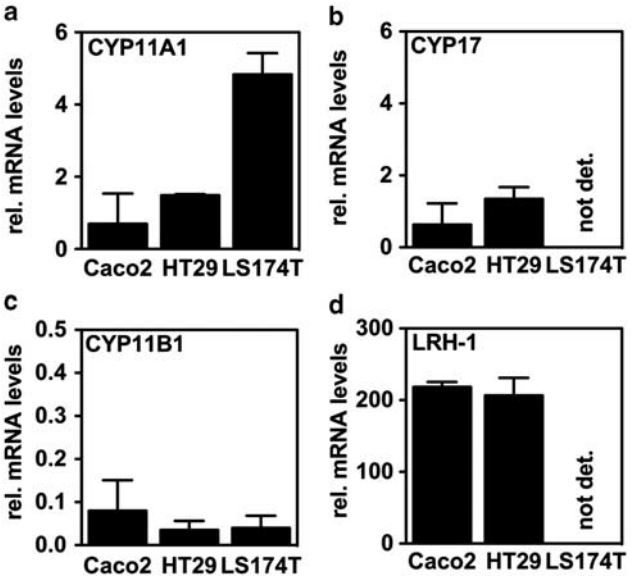

Figure 1 Colon carcinoma cell lines express steroidogenic factors. Detection of the steroidogenic enzymes *CYP11A1* (**a**), *CYP17* (**b**), *CYP11B1* (**c**) and *LRH-1* (**d**) by quantitative reverse transcription (RT)–PCR in the colon cancer cell lines Caco2, HT29 and LS174T. Mean values of triplicates ± s.d. of a typical experiment out of two are shown.

(Mueller *et al*., 2007). The expression of the steroid-producing enzyme machinery and the synthesis of cortisol in the adrenals are highly dependent on the nuclear factor steroidogenic factor-1 (SF-1, NR5A1; Parker *et al*., 2002). In contrast, SF-1 seems to be dispensable for steroid synthesis in the intestinal epithelium and is substituted by LRH-1. To assess the role of LRH-1 in steroidogenic enzyme expression in CRC cells, Caco2 cells were transiently transfected with an LRH-1 expression plasmid, and the promoter activity of the steroidogenic enzyme genes *CYP11A1*, *CYP17* and *CYP11B1* was analyzed by luciferase reporter assays (Supplementary Figure 1). Interestingly, Caco2 cells demonstrated an already very high basal *CYP11A1* and *CYP17* activity (fold induction over empty vector control), confirming the basal transcription of these enzymes seen in Figures 1a–d. Increasing amounts of LRH-1 expression plasmid dose-dependently induced the reporter constructs for these steroidogenic enzymes. Stimulation of the cells with phorbol ester phorbol 12-myristate 13-acetate (PMA) further enhanced the promoter activity of these genes, likely due to activation of LRH-1 transcriptional activity (Lee *et al*., 2006; Mueller *et al*., 2007). The stimulatory activity of PMA was confirmed when endogenous expression of steroidogenic enzymes was analyzed in Caco2 and HT29 cells. Treatment of cells with PMA induced *CYP11A1* and *CYP11B1* expression in Caco2 cells and *CYP11A1* expression in HT29 cells, whereas LRH-1 expression levels remained unchanged in both cell lines, in agreement with a PMA-induced activation of LRH-1 transcriptional activity (Supplementary Figure 2).

Expression of steroidogenic enzymes in CRC cell lines is dependent on endogenous LRH-1

To investigate whether endogenous LRH-1 regulates the basal expression of the steroidogenic enzyme in CRC cell lines, LRH-1 expression in HT29 and Caco2 cells was downregulated using RNA interference. Figure 2 shows that transfection of HT29 cells with LRH-1-specific small interfering RNA resulted in ~55% reduction of LRH-1 expression compared with control treated cells. Analysis of steroidogenic enzyme expression revealed that downregulation of LRH-1 resulted in reduced *CYP11A1*, *CYP17* and *CYP11B1* expression, indicating that LRH-1 is involved in the basal transcription of these steroidogenic enzymes. In contrast, the expression of an unrelated gene product, for example, retinoblastoma protein Rb, was not affected by LRH-1 downregulation. Similar findings were obtained with Caco2 cells (data not shown).

Colon cancer cell lines metabolize steroid precursors and produce cortisol

The basal and inducible expression of steroidogenic enzymes suggested that CRC cells are actually capable of producing cortisol. The culture supernatant of CRC cell lines was thus analyzed for the presence of cortisol by different techniques. In a first step, Caco2 and HT29 cells were cultured with ^{14}C-labeled progesterone and the conversion to ^{14}C-cortisol over time was analyzed by thin-layer chromatography. Starting after 2 h and further increasing over 24 h, a decrease in progesterone and a gradual accumulation of steroid intermediates was observed in both cell lines. Some of these products were identified as 17OH-progesterone and deoxycortisol. Critically, already after 2 h, cortisol was detectable in both cell lines, confirming that CRC cell lines are capable of producing GC (Supplementary Figure 3).

We next analyzed cortisol production in a quantitative manner. Caco2 cells were cultured in the presence or absence of the GC synthesis inhibitor metyrapone, and cortisol production in the cell-free supernatant was measured by radioimmunoassay. Interestingly, Caco2 cells secreted a considerable and readily detectable amount of cortisol into the supernatant. Cortisol synthesis was blocked with metyrapone, confirming the *de novo* synthesis of GC (Figure 3a). Intriguingly and in agreement with the steroidogenic enzyme expression pattern (Figures 1a–d), GC synthesis was not uniform for all colon cancer cell lines. Caco2 and HT29 cells were found to produce significant amounts of cortisol

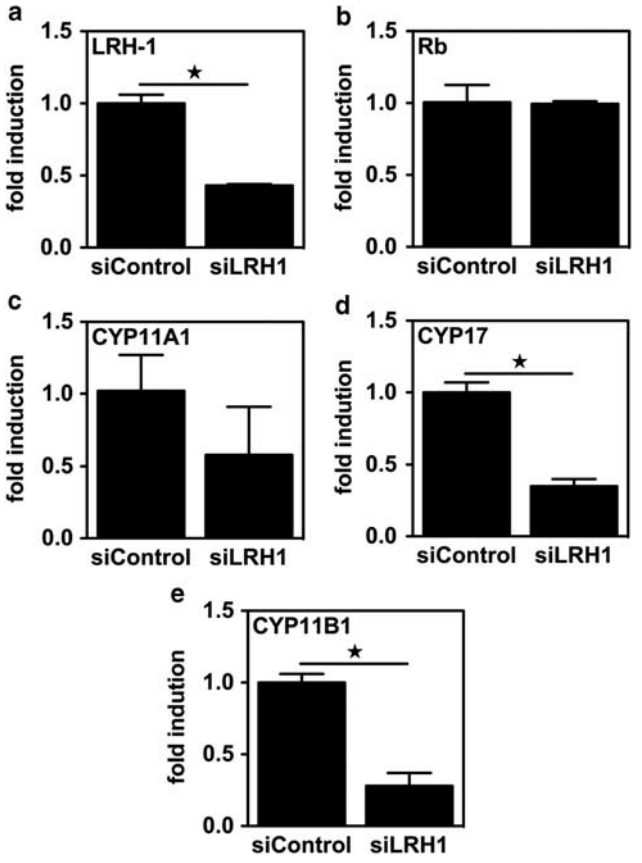

Figure 2 The expression of steroidogenic enzymes is dependent on endogenous LRH-1. HT29 cells were transfected with control small interfering RNA (siRNA) or siRNA for LRH-1, and the expression of LRH-1 (**a**), Rb (**b**), *CYP11A1* (**c**), *CYP17* (**d**) and *CYP11B1* (**e**) was measured by quantitative RT–PCR. Mean values of triplicates ± s.d. of a typical experiment out of three are shown. $^*P<0.05$.

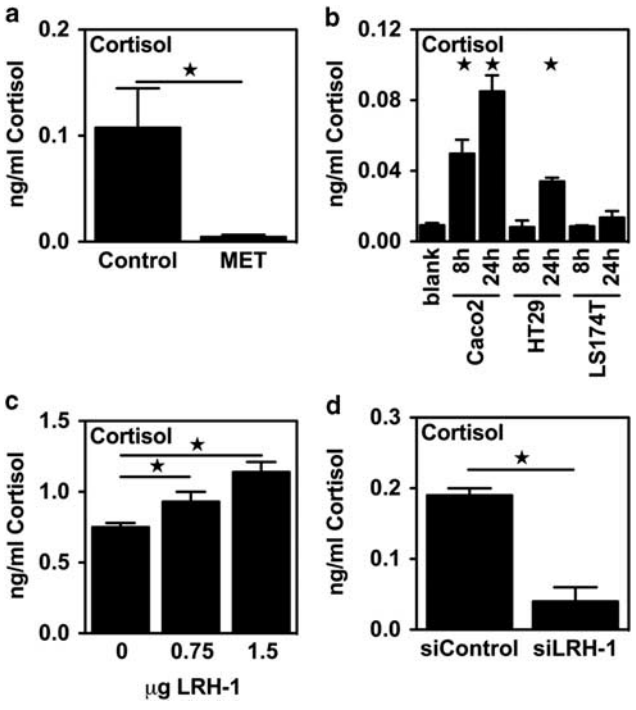

Figure 3 Colon cancer cells produce cortisol in an LRH-1-dependent manner. (**a**) Caco2 cells were cultured in the absence or presence of the GC synthesis inhibitor metyrapone (Met) for 24 h, and the presence of cortisol in the supernatant was measured by radioimmunoassay. (**b**) Caco2, HT29 and LS174T cells were cultured for 8 and 24 h, and cortisol in the supernatant was analyzed. (**c**) Caco2 cells were transfected with increasing amounts of LRH-1 expression plasmid, and cortisol production was measured. (**d**) Caco2 cells were treated with control siRNA (siControl) or LRH-1-specific siRNA (siLRH-1), and cortisol production was analyzed. $^*P<0.05$.

although no specific cortisol synthesis was detected in LS174T cells (Figure 3b).

We next investigated the role of LRH-1 in tumor cell-derived GC synthesis. A further increase in basal cortisol production was observed when Caco2 cells were transfected with a LRH-1 expression plasmid (Figure 3c). Furthermore, downregulation of LRH-1 using RNA interference resulted in a substantial reduction of basal cortisol synthesis (Figure 3d), indicating that LRH-1 is critically involved in the regulation of cortisol synthesis in CRC cell lines. The critical role of LRH-1 was further confirmed in LS174T cells. As shown in Figure 1b, LS174T cells do not express detectable levels of LRH-1 and only a restricted pattern of steroidogenic enzymes. Overexpression of LRH-1 in LS174T cells led to a strong induction of *CYP17* and *CYP11B1* expression and a substantial release of cortisol, indicating that LRH-1 is a master regulator of GC synthesis in CRC cells (Supplementary Figure 4).

The capacity of CRC cell lines to produce cortisol was also assessed in a GC bioassay. HEK 293T cells were transiently transfected with a GC receptor response element luciferase reporter construct and a GC receptor expression plasmid, as described elsewhere (Pazirandeh *et al*., 1999). Incubation with increasing concentrations of cortisol led to a dose-dependent induction of the reporter construct, that is, the GC receptor-dependent induction of luciferase activity. Similarly, supernatant of NCI-H295R (positive control), Caco2 and HT29 induced luciferase activity, reflecting a considerable GC bioactivity in the supernatant of CRC cells (Supplementary Figure 5). Critically, the GC bioactivity of all the three cell lines was completely blocked when cells were cultured with metyrapone. These findings clearly demonstrate that CRC cell lines produce bioactive GC.

Human CRC specimens express steroidogenic factors

We next aimed at confirming our data generated in CRC cell lines in primary colon carcinoma, and comparing them to the normal colonic mucosa. Tissue specimens from the center or the edge of CRC, and the normal mucosa were thus collected from 26 individual tumor patients, and the expression of steroidogenic factors was analyzed by quantitative reverse transcription–PCR (Figure 4). Interestingly, steroidogenic enzyme genes *CYP11A1* and *CYP11B1*, and regulatory factors such as LRH-1 and steroid acute regulatory protein (StAR) were detectable in the majority of the tissues analyzed. *CYP11B1*, StAR and LRH-1 were found to be expressed at higher levels either in the tumor edge or the tumor center, when compared with levels seen in normal mucosa, though differences in LRH-1 and StAR expression were not statistically significant (Figures 4b–d). StAR is involved in the delivery of cholesterol to the mitochondria and thus a prerequisite for effective steroid synthesis. In contrast, *CYP11A1* expression was in average significantly higher in the untransformed tissue compared with individual tumor samples (Figure 4a). Next to the relative expression, we also analyzed the frequencies of *CYP11A1*, *CYP11B1*, StAR and LRH-1 expression in tumor and normal tissue samples (Figures 4e–h). This revealed that *CYP11A1*, *CYP11B1* and StAR were generally more frequently expressed in tumor samples than in normal tissue, whereas LRH-1 was uniformly expressed in the majority of tumor and normal tissue samples.

The expression pattern of LRH-1 in normal colonic mucosa and CRC was also analyzed by immunohistochemistry. As reported previously, LRH-1 was found to be expressed in the nucleus and to be restricted to the intestinal crypts of the normal intestinal mucosa (Supplementary Figure 6A), in agreement with its

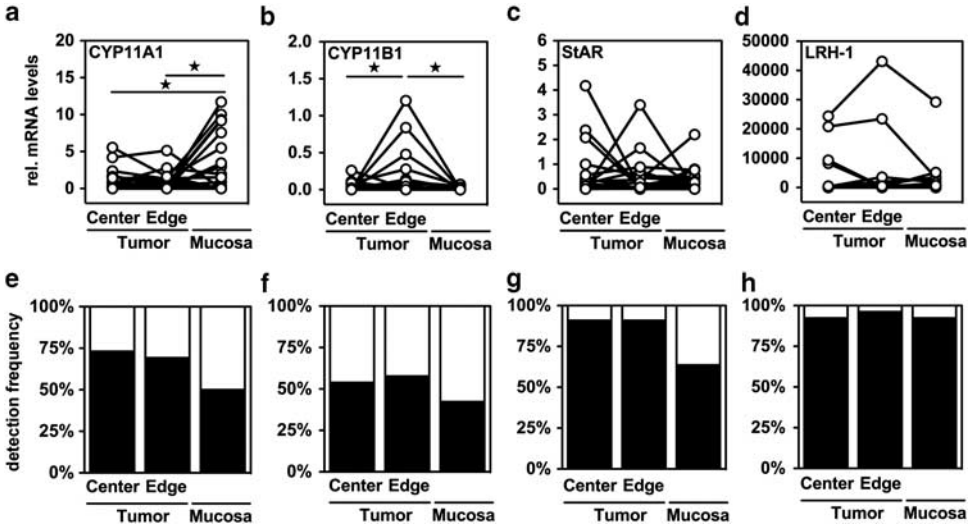

Figure 4 Primary human colorectal tumors express steroidogenic factors. Tissue specimens from colorectal tumor center, tumor edge and normal colonic mucosa of 26 patients were isolated and gene expression of *CY11A1* (**a**), *CYP11B1* (**b**), StAR (**c**) and LRH-1 (**d**) was measured by quantitative PCR. The relative expression levels in tissue samples are shown. Samples from individual patients are connected by lines (tumor center, tumor edge and normal mucosa). $*P<0.05$. (**e**–**h**) Shows the frequency of samples with detectable (black bar) versus undetectable (white bar) gene expression for *CY11A1* (**e**), *CYP11B1* (**f**), StAR (**g**) and LRH-1 (**h**).

important role in crypt cell proliferation and epithelial cell renewal (Botrugno *et al*., 2004; Schoonjans *et al*., 2005). Although LRH-1 also had a nuclear expression pattern in primary CRC specimens, it was expressed uniformly throughout the tumor (Supplementary Figure 6B). A similar finding was made in liver metastases of CRC (Supplementary Figure 6C).

Human colon cancer specimens produce bioactive GC ex vivo

To provide evidence for cortisol synthesis in primary human CRC tissue, we established tissue cultures of CRC specimens and normal colonic mucosa and analyzed the culture supernatant for the presence of GC using a GC bioassay. Interestingly, we could demonstrate that human colon cancer specimens produced bioactive GC, whereas normal mucosa lacked this capacity under basal conditions. Importantly, treatment of tissue cultures with metyrapone resulted in a clear reduction of GC bioactivity, indicating that GCs were produced *in situ*. GC synthesis in normal colonic tissue could be induced by treatment with PMA, whereas it could not be further increased in the CRC tissue (Figure 5), likely reflecting the pre-activated status of steroidogenesis in colon cancer cells.

Tumor cell-derived GCs suppress T-cell activation and induce apoptosis

Given the potent immunosuppressive activities of GC, we aimed at investigating whether CRC-derived GC can inhibit the activation of T lymphocytes. Murine splenic T cells were stimulated in the presence or absence of increasing doses of cortisol and the expression of the activation marker CD69 was assessed by flow cytometry. Cortisol treatment resulted in a substantially reduced T-cell activation and subsequent expression of the activation marker, which could be reversed by treatment of cells with the GC receptor antagonist RU-486 (Figure 6a; Cima *et al*., 2004, 2006). Culture of T cells with the supernatant from Caco2 cells resulted in a substantial inhibition of activation-induced CD69 expression, which was blocked by RU-486, confirming the presence of bioactive GC in the supernatant (Figure 6b). As GCs are also potent inducers of apoptosis, we also tested the activity of GC in the Caco2 cell culture supernatant on the cell death induction in murine thymocytes. Figures 6d–e show that GC in the tumor cell culture supernatant induced thymocyte apoptosis in a GC receptor-dependent manner.

We next analyzed the supernatant of primary CRC tumor culture for the presence of immunoregulatory

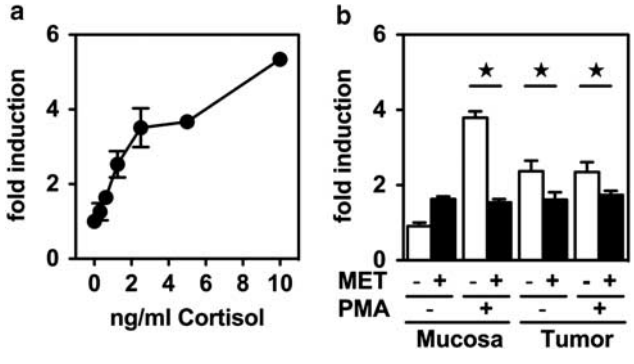

Figure 5 Primary human colon cancer tissue produces bioactive GC. Tissue specimens from normal colonic mucosa or primary CRC (tumor) were cultured *ex vivo* in the presence or absence of the GC synthesis inhibitor metyrapone, and stimulated with medium control or 3 ng/ml PMA. GCs released into the cell-free supernatant were measured using bioassay as described in Materials and methods. (**a**) Bioactivity induced by a standard curve of cortisol. (**b**) Detection of GC bioactivity in the culture supernatant of normal mucosa and colorectal tumor tissue. (■ metyrapone-treated tissue, □ control treated tissue). Mean values of triplicates ± s.d. of a typical experiment out of two are shown. $*P<0.05$.

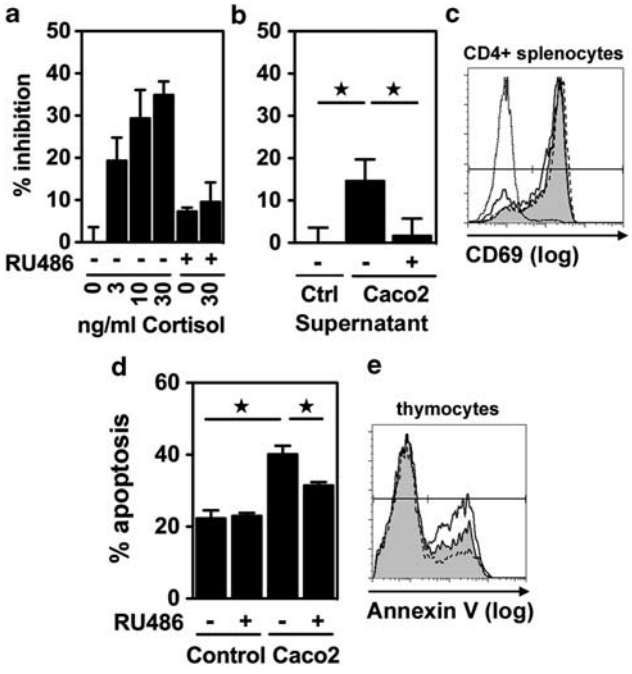

Figure 6 Tumor cell-derived GCs suppress T-cell activation and induce apoptosis. (**a**) Splenic T cells were activated in the presence of increasing concentrations of cortisol, and CD69 expression on $CD4^+$ T cells was measured. GC receptor specificity was confirmed by blocking with 100 nM RU486. (**b**) Splenic T cells were activated in the presence of medium control or heat-inactivated culture supernatant from Caco2 cells. GC activity was blocked by addition of 100 nM RU486. Mean values of triplicates ± s.d. of a typical experiment out of three are shown. Statistical differences in T-cell activation were compared with that of the respective control. $*P<0.05$. Cells were exposed to cortisol standard or culture supernatant for 6 h and then stimulated for 8 h. (**c**) CD69 expression profiles of activated $CD4^+$ T cells (solid gray histogram), treated with supernatant from Caco2 cells (solid line), treated with supernatant from Caco2 cells plus RU486 (dashed line). The dotted line shows isotype control staining. (**d**) Murine thymocytes were cultured with control medium or with culture from Caco2 cells, in the presence or absence of RU486, and apoptosis was assessed after 8 h by Annexin V staining. Mean values of triplicates ± s.d. of a typical experiment are shown ($n=3$). $*P<0.05$. (**e**) Histograms of Annexin V staining of control-treated thymocytes (dashed line), Caco2 supernatant-treated cells (solid line), and Caco2 supernatant- and RU486-treated cells (solid gray histogram).

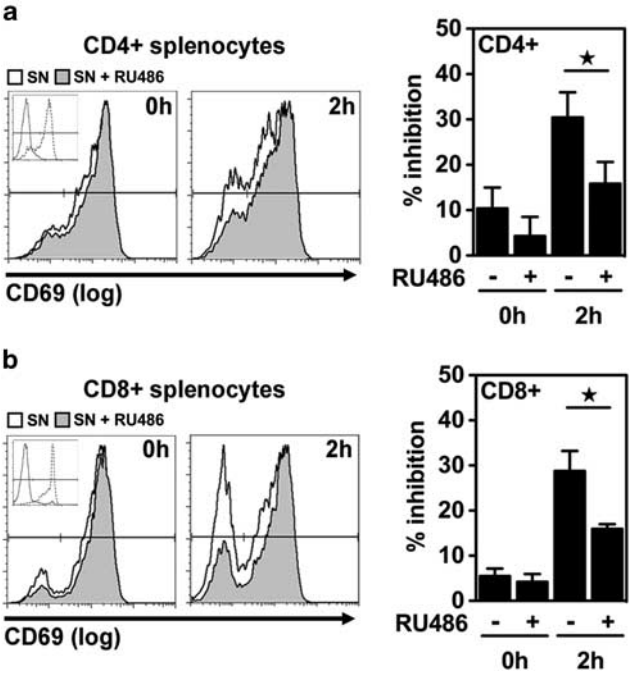

Figure 7 Primary CRC release immunoregulatory GC. Primary CRC tumor tissue was cultured for 0 and 2 h, and cell-free sterile filtered supernatant was harvested. Splenic T cells were activated and exposed to the tumor culture supernatant, in the presence or absence of the GC receptor antagonist RU486. CD69 expression on activated CD4+ (**a**) and CD8+ (**b**) T cells were analyzed. The left panels show examples for typical CD69 expression profiles by flow cytometry, the right panels show quantitative data of culture supernatant-mediated inhibition of CD69 expression (% inhibition relative to stimulated splenocytes without tumor cell supernatant). Inserts show isotype control staining (solid line) and CD69 staining (doted line) of activated T cells.

GC. Supernatant from tumor samples was harvested directly or after 2 h, and exposed to stimulated murine T cells. CD69 expression in $CD4^+$ and $CD8^+$ T-cell subsets was analyzed. Figure 7 shows that the expression of CD69 in $CD4^+$ (Figure 7a) and $CD8^+$ T cells (Figure 7b) was substantially reduced when cells were exposed to the culture supernatant of primary CRC tumors. Importantly, this inhibition could be reversed by blocking the GC receptor with RU486 (Cima *et al*., 2004, 2006).

Discussion

While the orchestrated activation of cells of the innate and adaptive immune system is essential for the defense of the body from invasion by potentially harmful pathogens, their uncontrolled action often leads to tissue damage and loss of vital organ functions. Therefore, different tissues and organs have developed mechanisms that limit uncontrolled immune responses and associated tissue damage, and thereby become 'immunologically privileged'. This is particularly evident in organs that are essential for survival or reproduction. For example, the eye secretes the immunoregulatory cytokine transforming growth factor-β and inhibits local immune cell activation (Chen *et al*., 1998; Wahl *et al*., 2006). In addition, Fas ligand is abundantly expressed in the retina and cornea, and can induce apoptosis in infiltrating leukocytes during inflammatory reactions (Griffith and Ferguson, 1997). Various tumors are even better known for displaying a plethora of mechanisms that help them to escape from the prosecution by the immune system. These include the secretion of immunosuppressive cytokines, such as interleukin-10 and transforming growth factor-β, reduced antigen presentation by downregulation of MHC molecules, conditioning of the environment and immune cells, and the expression of pro-apoptotic molecules, such as Fas ligand and TRAIL (Igney and Krammer, 2002). This altogether leads to a 'stealth effect', which helps the tumor to limit or avoid destruction by cytotoxic lymphocytes.

In this study, we now show a novel and so far unrecognized potential mechanism of immune evasion by colon carcinomas via the synthesis and release of bioactive immunosuppressive GC. We found that colon carcinoma cell lines as well as primary tumors express the enzymatic steroidogenic machinery, produce cortisol and can regulate T-cell activation. Although steroid production has been previously shown in tumors derived from primary steroidogenic organs, such as adrenals, testis and ovaries, our present study demonstrates for the first time the synthesis of cortisol by a tumor derived from a non-endocrine tissue. Furthermore, we provide evidence that CRC-derived GC can regulate the activation of immune cells and thereby could potentially contribute to tumor immune evasion.

Of interest is the fact that some but not all the tested colon carcinoma cell lines expressed the majority of enzymes required for the *de novo* synthesis of cortisol. The expression pattern directly correlated with the capacity of these CRC cell lines to produce cortisol. This may reflect a previous selection process *in vivo*, where the synthesis of immunoregulatory GC may have given the tumor a certain advantage over non-steroid-producing tumor cells. Although the analysis of steroidogenic factor expression in primary tumors revealed a rather heterogeneous picture, with high and low expressors, the release of immunoregulatory GC into the tumor environment could significantly limit anti-tumor immune responses and favor tumor growth. In line with this notion are recent findings demonstrating that the degree of tumor infiltration by memory $CD8^+$ T cells strongly correlates with the overall survival of CRC patients (Pages *et al*., 2005; Galon *et al*., 2006), indicating that anti-tumor immune responses are able to significantly limit tumor growth. Clearly, tumors that can limit or prevent anti-tumor immune responses will develop more aggressively and will cause a more rapid death of the patient. GC synthesis by CRC, as demonstrated in this study, may substantially contribute to these processes.

We have previously identified the nuclear receptor and transcription factor LRH-1 as a critical regulator of intestinal GC synthesis (Mueller *et al*., 2006, 2007;

Coste *et al*., 2007). Also, in human colon carcinoma cell lines, LRH-1 was found to have an essential role in the transcriptional regulation of steroidogenesis. Down-regulation or inhibition of LRH-1 in CRC cell lines significantly reduced the expression of steroidogenic enzymes and the ability to produce cortisol. Interestingly, LRH-1 seems to have at least a dual role in CRC. While we here clearly show that LRH-1 has a prominent function in the regulation of GC synthesis, previous findings also demonstrated that LRH-1 regulates the expression of cell-cycle proteins (Botrugno *et al*., 2004) and thereby might be involved in the development of intestinal tumors (Schoonjans *et al*., 2005). As LRH-1 haplo-insufficient mice have reduced tumor development in two different models of intestinal tumor formation, it is likely that LRH-1 contributes to CRC development via the regulation of cell-cycle progression, in addition to the here-proposed function in tumor immune escape via the synthesis of GC. In support of this notion is a recent finding that the LRH-1 antagonist small heterodimer partner (SHP) is frequently down-regulated in hepatocellular carcinoma by epigenetic silencing, which is associated with increased tumor growth (He *et al*., 2008). Also, in CRC uniform LRH-1 expression was frequently observed, whereas in the normal mucosa it remained restricted to the proliferating cells of the crypts.

In summary, we demonstrated for the first time that CRC cell lines and primary tumor specimens express steroidogenic factors and produce immunoregulatory GC. Our findings further suggest that tumor-derived GC may modulate or suppress anti-tumor immune responses and thereby contribute to tumor immune escape. Given the important role of LRH-1 in both tumor cell-cycle progression and GC synthesis, the specific inhibition of this nuclear receptor and transcription factor may become an interesting target in CRC therapy.

Materials and methods

Cells and reagents

The human embryonic kidney fibroblast cell line 293T (CRL-11268), the human colon cancer cell lines Caco2 (ATCC HTB-37), HT29 (ATCC HTB-38), T84 (ATCC CCL-248), Colo205 (ATCC CCL-222) and LS174T (ATCC CCL-188), and the human adrenal cancer cell line NCI-H295R (ATCC CRL-2128) were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in Isocove's modified Dulbecco's medium containing 10% fetal calf serum, 2 mM glutamine and 50 μg/ml gentamycin at 37 °C and 5% CO_2. All reagents were from Sigma-Aldrich (Buchs, Switzerland), unless stated otherwise.

Plasmids

The luciferase reporter constructs for the genomic promoter sequences of *CYP11A1*, *CYP17*, *CYP11B1*, as well as the expression plasmids for β-galactosidase, have been described previously (Mueller *et al*., 2006, 2007; Kempna *et al*., 2007; Atanasov *et al*., 2008). The reporter plasmid GRE2tk and the expression plasmid for GC receptor SVGR1 were kindly provided by Sam Okret (Karolinska Institute, Stockholm, Sweden; Pazirandeh *et al*., 1999). The expression plasmids for human LRH-1 was kindly provided by Kristina Schoonjans (Ecole Polytechnique Federale de Lausanne EPFL, Lausanne, Switzerland).

Detection of steroidogenic enzyme genes and LRH-1 mRNA by quantitative reverse transcription–PCR

Tissue specimens were obtained from patients undergoing surgery for primary CRC at the Department of Visceral Surgery and Medicine, Insel University Hospital, University of Bern, Switzerland. All patients provided informed consent and experiments were reviewed by an institutional review board. Tissue samples from the tumor center, the tumor edge and non-cancerous normal mucosa, ~10 cm from the tumor edge, were collected. Tissues and CRC cell lines were homogenized in RNA isolation reagent, RNA was isolated and complementary DNA using a HighCapacity RT reaction kit (Qiagen (Hombrechtikon, Switzerland). The expression of *CYP11A1*, *CYP11B1*, as well as StAR and LRH-1 was analyzed by quantitative PCR on an Applied Biosystems Real-time PCR 7500 machine using SYBR green and Quantitect primer assays (Qiagen). Gene expression was normalized by GAPDH RNA levels and plotted as relative mRNA levels.

Luciferase reporter assays

Luciferase reporter constructs and β-galactosidase expression vector for transfection control were transiently transfected into Caco2 cells by lipofection. The amount of total transfected DNA was normalized with the corresponding empty vector plasmid. At 24 h after transfection, cell lysates were assessed for luciferase and β-galactosidase activity as previously described (Mueller *et al*., 2007; Atanasov *et al*., 2008). In some experiments, cells were stimulated with PMA (Calbiochem) as indicated for 16 h before luciferase assay. Values were calculated as fold induction over activity of the corresponding control vector and plotted as mean ± s.d.

RNA interference

Caco2 and HT29 cells were plated in six-well plates. Cells were transfected with 10 nM small interfering RNA for LRH-1 (siLRH-1) or control, small interfering RNA (siControl; Dharmacon ON-TARGET plus SMARTpool, Thermo Fisher Scientific, Lafayette, CO, USA) using HiPerfect transfection reagents (Qiagen). This treatment was repeated after 24 h. After 48 h, cells were harvested and mRNA expression levels for LRH-1 and steroidogenic enzymes were assessed by quantitative PCR.

Ex vivo *tissue culture of tumor and mucosal tissue*

Cancerous and non-malignant human colon tissue samples were cut into small pieces (<2 mm diameter), washed in phosphate-buffered saline, distributed equally in 24-well plates and incubated with Isocove's modified Dulbecco's medium containing 10% steroid-free fetal calf serum at 37 °C. In some experiments, GC synthesis was either stimulated by adding PMA or blocked by addition of 200 μg/ml metyrapone to the organ culture as previously described (Cima *et al*., 2004). After 2–16 h, supernatant was harvested, heat inactivated and *ex vivo* GC synthesis was analyzed.

Detection of steroidogenesis by thin-layer chromatography

Caco2 and HT29 cells were cultured in six-well plates and 200 000 c.p.m. ^{14}C-labeled progesterone ([4-^{14}C]-progesterone, 55 mCi/mmol, American Radiolabeled Chemicals Inc., St Louis, MO, USA) was added per well. Cell supernatant

was harvested at various time points and steroids were extracted as described elsewhere (Dardis and Miller, 2003). Samples were then separated by thin-thin-layer chromatography using methylene chloride:methanole:H_2O (200:30:1) as solvent system. Steroids were visualized on a Storm PhosphorImager (GE Healthcare, Little Chalfont, UK). The identity of the radioactive steroid spots was confirmed by co-chromatography, with steroid standards as indicated.

GC bioassay
HEK 293T cells were transiently transfected with GRE2kt GC receptor reporter plasmid and the SVGR1 GC receptor expression plasmid using the calcium phosphate precipitation method. Then, heat-inactivated supernatant from CRC cell lines or *ex vivo* tumor cultures was added as indicated for 8 h. Cortisol titration was used as standard. Reporter cells were lysed and luciferase activities were determined. Values were calculated as fold induction over basal promoter activity and were plotted as mean ± s.d.

Cortisol radioimmunoassay
Colon cancer cell lines were plated in six-well plates and were allowed to adhere. In some experiments, LRH-1 was either silenced by small interfering RNA-mediated gene knock down or overexpressed as described above. Then, medium was changed and incubated for the indicated time points. Cortisol concentrations in culture supernatant from cell lines were measured by radioimmunoassay using an adapted commercial cortisol assay (C8409, Sigma-Aldrich). Cortisol concentration was calculated from a cortisol standard curve and plotted as mean ± s.d.

T-cell activation assay
Murine splenocytes from a C57Bl/6 mouse were incubated with standard concentrations of cortisol or heat-inactivated supernatant from Caco2, HT29 or NCI-H295R cells. In some experiments, splenocytes were pre-incubated with the GC receptor antagonist 100 nM RU486 (Brunner *et al*., 2001). After 2 h, cells were stimulated with 1 μg/ml concanavalin A for 20 h, and CD69 expression on $CD4^+$ T cells (antibodies from eBiosciences, San Diego, CA, USA) was detected by flow cytometry.

Apoptosis induction thymocytes
Thymocytes were isolated from 7-week-old CL57/BL6 mice and cultured in 96-well plates in the presence of control medium, cortisol standard or supernatant from 8 h Caco2 tumor cell culture. In some experiments, GC action was antagonized by adding 30 ng/ml RU486. Cells were harvested after 16 h and apoptosis was measured by Annexin V binding and flow cytometry.

Statistical analysis
Statistical analyses were done using Prism 5 software. Student's *t*-test was used for the comparison of two independent groups, analysis of variance test with Bonferroni correction for the comparison of three and more independent groups. Values <0.05 were considered significant.

Abbreviations

CRC, colorectal carcinoma; CYP, cytochrome P450; GC, glucocorticoids; LRH-1, liver receptor homolog-1/NR5A2; SF-1, steroidogenic factor-1/NR5A1; StAR, steroid acute regulatory protein.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)