Myeloid STAT3 promotes formation of colitis-associated colorectal cancer in mice

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Abbreviations: AOM, Azoxymerthane, CRC, colorectal cancer; DSS, Dextran sulfate; IHC, immunohistochemistry; STAT3, signal transducer and activator of transcription 3; TAM, tumor-associated macrophage

Myeloid cells lacking STAT3 promote antitumor responses of NK and T cells but it is unknown if this crosstalk affects development of autochthonous tumors. We deleted STAT3 in murine myeloid cells (STAT3<sup>-/-</sup>) and examined the effect on the development of autochthonous colorectal cancers (CRCs). Formation of Azoxymerthane/Dextran sulfate (AOM/DSS)-induced CRCs was strongly suppressed in STAT3<sup>-/-</sup> mice. Gene expression profiling showed strong activation of T cells in the stroma of STAT3<sup>-/-</sup> CRCs. Moreover, STAT3<sup>-/-</sup> host mice were better able to control the growth of transplanted MC38 colorectal tumor cells which are known to be killed in a T cell-dependent manner. These data suggest that myeloid cells lacking STAT3 control formation of CRCs mainly via cross activation of T cells. Interestingly, the few CRCs that formed in STAT3<sup>-/-</sup> mice displayed enhanced stromalization but appeared normal in size indicating that they have acquired ways to escape enhanced tumor surveillance. We found that CRCs in STAT3<sup>-/-</sup> mice consistently activate STAT3 signaling which is implicated in immune evasion and might be a target to prevent tumor relapse.

Introduction

CRC represents the third most common form of cancer in humans. CRC develops frequently in the context of inflammatory bowel disease demonstrating a tumor-promoting role of inflammation. Moreover, stroma immune cells support growth of CRC via production of cytokines that activate the oncogenic transcription factors NFκB and STAT3. However, the stroma also contains immune cells with antitumor activities which force tumors to develop mechanisms of escape and immune suppression. One paradigm is the ability of tumors to modulate polarization of TAMs. Th1 cytokines polarize macrophages toward the M1 phenotype which is responsible for innate immune attack of infectious pathogens but also neoplastic cells. Alternative activation by Th2 cytokines polarizes macrophages toward the M2 phenotype which is characterized by low expression of M1 markers (e.g., iNOS) but high expression of M2 markers (e.g., Arginase-1). M2 macrophages have reduced antitumorigenic activities but promote angiogenesis and tumor progression. Repolarization of M2 macrophages toward the M1 phenotype in tumors represents a promising option for antitumor therapies.

Another layer of complexity was recently discovered in breast tumors where an additional TAM population, distinguishably from M1 or M2 polarized macrophages, was identified. STAT3 phosphorylation at tyrosine residue 705 (pY-STAT3) via Janus kinases (JAKs) is induced by pro- and anti-
inflammatory cytokines such as IL-6 and IL-10. The oncogenic role of STAT3 in CRC tumor cells has been demonstrated in several studies. However, STAT3 seems to have a suppressive role in advanced CRCs because ApcMin mice lacking STAT3 in tumor cells developed aggressive intestinal carcinomas that were not observed in ApcMin control animals. STAT3 activation in tumor cells represents an important immune escape mechanism as cancer cells exploit STAT3-induced expression of VEGF and IL-10 to blunt maturation of stroma DCs. An immune suppressive action is further given by the STAT3-dependent repression of pro-inflammatory cytokines and chemokines including IFNγ, TNFα, IL-6, and CXCL10.

Apart from tumor-cell specific functions, STAT3 is considered to promote tumor formation when being activated in stroma cells of CRCs. Tumor transplantation experiments into mice lacking STAT3 in the hematopoietic system demonstrated that STAT3 activation in immune cells prevents antitumorogenic activities of DCs, Th1 cells, NKs, and neutrophils. This led to the concept of inhibiting stroma/hematopoietic STAT3 as potential strategy for cancer immunotherapy. Myeloid STAT3 is considered of particular importance for the modulation of Th1-like antitumor responses. Consequently, inhibition of STAT3 in myeloid cells and B cells using CpG-STAT3 siRNA enhanced cytotoxic T cell (CTL)-mediated responses in a melanoma transplantation model.

Here, we demonstrate that ablation of myeloid STAT3 significantly interfered with development of autochthonous AOM/DSS-induced colorectal tumors in mice. This effect was mediated by an enhanced antitumor T cell response. Weighted gene co-expression network analysis (WGCNA) uncovered sustained T cell-mediated immune surveillance in escaped STAT3Δm tumors accompanied by enhanced STAT3 signaling in tumors which might promote immune evasion.

Results

Myeloid STAT3 promotes formation of colorectal tumors

We investigated the role of STAT3 in the myeloid stroma cells of autochthonous colorectal tumors developing under inflammatory conditions using mice with conditional inactivation of STAT3 in myeloid cells (STAT3Δm = LysMCre/Cre STAT3floX/floX) and corresponding controls (STAT3wt = LysMCre/Cre STAT3+/+). The LysMCre knock-in alleles were kept homozygous to account for potential Cre or LysM knock-in effects. Successful deletion of STAT3 in myeloid cells was confirmed by Western blot of granulocyte-macrophage precursors, FACS-sorted peritoneal macrophages (Fig. S1A) and qPCR of FACS-sorted splenic macrophages (Fig. S1B). Histopathological analysis and IHC-staining revealed no major changes of cell differentiation or proliferation in colon and small intestine of STAT3Δm mice (Fig. S2A). Despite the observation that deletion of STAT3 in myeloid cells provokes Th1 differentiation and development of colitis, we could not detect severe colitis in STAT3Δm mice in the C57BL/6 genetic background (Fig. S2B). We only observed a consistently increased mean fluorescence intensity of the activating protein CD69 on CD4+ T cells in the lamina propria of DSS-treated mice.
STAT3Δm mice (Fig. S3) indicative for Th1 differentiation.

To investigate the impact of myeloid STAT3 in autochthonous tumors, we induced CRC using AOM/DSS (Fig. 1A). Tumor multiplicity was strongly reduced in STAT3Δm male mice but tumor size was not affected (Fig. 1B). Tumor analysis in female mice gave similar results (data not shown). Tumor parameters such as cell proliferation and cell survival were unaltered in STAT3Δm CRCs (Fig. 1C) whereas increased numbers of blood vessels were found in the tumor stroma (Fig. 1D).

Myeloid STAT3 regulates stromalization of CRCs

Tumor progression requires the reciprocal interaction between tumor and stroma cells. Remarkably, histopathologic and immunohistochemical examination revealed an increased stromalization of STAT3Δm CRCs (Fig. 2A). Histomorphometric quantitation of IHC-stained sections (Fig. S4) demonstrated that the relative percentages of most cell types were unaltered in the stroma of STAT3Δm CRCs (Fig. 2B). Interestingly, RORγt+ Th17 cells were almost absent (Fig. 2B). Among macrophages, we found a reduction of cells expressing the macrophage M2 polarization protein Arginase-1 paralleled by an increase in the number of M1 expressing iNOS-positive cells (Fig. 2C).

Increased stromalization is frequently associated with enhanced tumor progression and invasiveness. However, no significant alteration in the percentages of low grade tumors, high grade tumors and carcinomas was observed in STAT3Δm mice (Fig. 2D).

Myeloid STAT3 is not required for NK cell-mediated immune surveillance of CRCs

Loss of STAT3 in myeloid cells leads to an aberrant activation of NK and T cells.18,25 Thus, we studied whether reduced colorectal tumorigenesis is caused by an improved immune surveillance exerted by NK or T cells. NK cell-dependent cytotoxicity was assessed ex vivo using spleen-derived NK cells and CFSE-stained YAC-1 target cells. No significant differences in killing efficacies of NK cells isolated from STAT3wt or STAT3Δm mice were

Figure 2. Enhanced stromalization of STAT3Δm tumors. (A) H&E staining (upper panel; T: tumor; S: stroma; scale bar indicates 100 μm) and double IHC for PDGFR/FSP1 (lower panel; scale bar indicates 50 μm) revealed increased stromal area in STAT3Δm tumors. FSP1+ fibroblasts are indicated by arrowheads. (B) IHC stainings with antibodies for indicated antigens were used for quantitative histomorphometry to define the composition of the tumor stroma. Bars represent data +/− SEM of n = 9 tumors in ≥ 3 animals per genotype. (C) STAT3Δm tumors displayed reduced percentages of Arginase-1+ cells and increased percentages of iNOS+ cells. Bars represent data +/− SEM of n ≥ 9 tumors in ≥ 3 animals per genotype. (D) Histopathological grading of colon tumors revealed no significant change in STAT3Δm mice (l.g.: low grade; h.g.: high grade; n = 97 STAT3wt and 26 STAT3Δm tumors).
rescence intensity of the activating receptor NKG2D in NK cells expressing the activating receptor Ly49D, which recognizes MHC-I alloantigens (Fig. S5B). The only consistent changes were lower numbers of NK cells but increased mean fluorescence intensity (MFI) of NKG2D on CD3+ Nkp46+ gated Ly49D+ NK cells but increased mean fluorescence intensity (MFI) of NKG2D on CD3+ Nkp46+ gated NK cells in STAT3Δm mice (n ≥ 4 mice per genotype). (D) Tumor weight and tumor images after transplantation of BCR/ABLp185 tumor cells into STAT3m and STAT3Δm host mice (n = 15 tumors in STAT3wt and 11 tumors in STAT3Δm mice). (E) STAT5ΔNK mice were employed as genetic model for NK ablation. The reduction of NK cell numbers in the intestine was confirmed by FACS analysis of mucosal cell preparations for CD45+ CD3+ CD19+ Nkp46+ NK cells (left graph). Reduction of NK cell numbers did not affect tumor multiplicity and mean tumor area of AOM/DSS-induced CRCs (n = 8 STAT3wt and 11 STAT3Δm mice). Bars represent data +/- SEM.

We next investigated the impact of NK cell-mediated immune surveillance on formation of autochthonous CRCs. For that purpose, we treated STAT5wt and STAT5ΔNK mice with AOM/DSS. STAT5ΔNK mice represent a model for genetic ablation of NK cells.27 However, no significant change in AOM/DSS-induced tumor formation was observed in STAT5ΔNK mice despite of the substantial reduction of Nkp46+ cell numbers in the gut (Fig. 3E). These data suggest that NK cell-mediated killing activities do not significantly contribute to immune surveillance of AOM/DSS-induced CRCs.

Myeloid STAT3 is required for T cell-mediated immune surveillance of CRCs

Apart from NK cells, CTLs and helper T cells are key determinants of tumor immune surveillance. Therefore, we performed in vivo cytotoxicity assays. m-TRP2181-188-immunized STAT3Δm mice displayed a slight but significantly enhanced killing activity of m-TRP2181-188-pulsed CFSE-labeled splenocytes in vivo (Fig. 4A) indicating enhanced CTL activity. To address whether the enhanced T cell-dependent cytotoxicity extends to CRCs, we transplanted isogenic colorectal MC38 adenocarcinoma cells. Remarkably, MC38 tumor growth, which is controlled by T cells,28 was significantly impaired in STAT3Δm host animals (Fig. 4B). Reduced tumor growth was accompanied by higher numbers of CD4+ T cells and slightly increased numbers of CD8+ cytotoxic T lymphocytes in tumor cell preparations (Fig. 4C). The number of immature myeloid-derived suppressor cells (MDSCs) was also elevated in tumors of STAT3Δm host mice whereas TAMs were reduced (Fig. 4C). No changes of NK cell infiltration were observed (data not shown). Moreover, NKG2D ligand RaeI and MHC-I protein expression levels were similar on MC38 tumor cells that developed in STAT3wt and STAT3Δm mice (data not shown).
These data demonstrate enhanced T cell-mediated killing of MC38 colorectal tumors cells in STAT3\textsuperscript{Dm} host mice. We next investigated if enhanced T cell activity is also evident in the stroma of autochthonous AOM/DSS-induced STAT3\textsuperscript{Dm} CRCs. Tumor and stroma tissue were separated by microdissection and microarray analysis was performed with isolated RNA. Genes indicative for T cell activation were upregulated in the stroma of STAT3\textsuperscript{Dm} CRCs (Fig. 4D). In human CRCs, a gene expression profile called Immunologic Constant of Rejection (ICR) signature\textsuperscript{29} is indicative for T cell activation and better prognosis of patients. Several genes of the human ICR signature such as stat1 were also found upregulated in murine STAT3\textsuperscript{Dm} CRCs (Fig. 4E). STAT1 is a key factor in immune surveillance\textsuperscript{30} and it has been shown that deletion of STAT3 shifts gp130-type cytokine signaling from a STAT3 to a STAT1 response.\textsuperscript{31} Consistently, increased numbers of stroma cells with STAT1 protein expression and STAT1 activation were detected in STAT3\textsuperscript{Dm} CRCs (Fig. 4F). Taken together, these data support the concept that CRC growth in STAT3\textsuperscript{Dm} mice is limited by T cell-mediated immune surveillance.

**Figure 4.** Characterization of cytotoxic T cell activity in STAT3\textsuperscript{Dm} mice. (A) In vivo cytotoxicity assay in STAT3\textsuperscript{wt} and STAT3\textsuperscript{Dm} mice immunized with m-TRP2181-188. CFSE\textsuperscript{low} target cells were used as internal control. CFSE\textsuperscript{high} target cells were loaded with an irrelevant peptide or m-TRP2181–188 (relevant peptide). Specific killing was calculated after FACS analysis of CFSE-labeled target cells (n = 4 mice per group). (B) Tumor weight of transplanted MC38 tumor cells into STAT3\textsuperscript{wt} and STAT3\textsuperscript{Dm} mice (n = 12 tumors in STAT3\textsuperscript{wt} and 14 tumors in STAT3\textsuperscript{Dm} mice). (C) FACS analysis of immune cell preparations revealed increased numbers of CD4\textsuperscript{+} T cells, MDSCs (Gr1\textsuperscript{hi} CD11b\textsuperscript{+}) and reduced numbers of TAMs (Gr1\textsuperscript{lo} CD11b\textsuperscript{+}CD11c\textsuperscript{lo}) in MC38 tumors of STAT3\textsuperscript{Dm} hosts. Bars represent data ±/SEM. (D) Affymetrix RNA expression data of laser-capture microdissected stroma tissue from STAT3\textsuperscript{wt} and STAT3\textsuperscript{Dm} CRCs for genes indicative for T cell activation (left graph) and IL-2 receptors (right graph). (E) Affymetrix RNA expression data of ICR genes measured in the tumor stroma. (F) STAT1 protein expression and activation in the stroma of STAT3\textsuperscript{Dm} CRCs. Images show IHC-stained tissue sections of stroma cells positive for phosphorylated (activated) pY-STAT1 (arrowheads). Scale bar indicates 50 μm. Corresponding IHC stainings were used for histomorphometric quantitation of STAT1- and pY-STAT1-positive cells in the tumor stroma (bar diagrams). Bars represent data ±/SEM of n ≥ 9 tumors in ≥ 3 animals per genotype.
Cell-autonomous changes of putative immunoediting pathways in STAT3<sup>Δm</sup> CRCs

Despite maintained T cell activation in the stroma, tumors of STAT3<sup>Δm</sup> mice did not show a significant reduction in size (Fig. 1B). This indicates that established CRCs in STAT3<sup>Δm</sup> mice must have developed strategies to escape the enhanced tumor surveillance. We thus investigated the microarray expression data of the microdissected tumor cell tissue separately for processes involved in immunoediting. RNA expression of immunosuppressive tumor-derived factors (Fig. S8A) and NKG2D stress-induced ligands (Fig. S8B) were unchanged. Unaltered protein expression of the NKG2D ligand RaeI was confirmed by IHC (data not shown). As STAT3<sup>Δm</sup> CRCs displayed increased expressing of complement factors in the stroma (Fig. 5A; “adaptive immune response”) we investigated expression of mCRPs, which protect tumor cells from complement attack. No differences were observed (Fig. S8C).

We next used the Molecular Signature Database of the Broad Institute to identify differentially regulated signaling pathways in tumor cells. Genes upregulated in STAT3<sup>Δm</sup> tumor cells (>2-fold) overlapped with genes upregulated in colonic tissue after treatment with IL-22 (a potent inducer of STAT3)<sup>32</sup> indicating enhanced STAT3 signaling. Since STAT3 activation is a prominent mechanism involved in immune escape of tumor cells,<sup>33</sup> we stained STAT3<sup>Δm</sup> tumors for STAT3 and activated nuclear pY-STAT3. Consistent with the overlap analysis, elevated levels of STAT3 and activated pY-STAT3 were evident in STAT3<sup>Δm</sup> tumor cells (Fig. 5B). The overlap analysis also suggested increased expression of c-Myc, a STAT3 target gene,<sup>34</sup> in STAT3<sup>Δm</sup> tumor cells which was confirmed by IHC (data not shown). In contrast, no obvious change in nuclear β-Catenin, the major driver of CRC formation, was observed (Fig. 5B). These data suggest that STAT3<sup>Δm</sup> CRCs activate STAT3 signaling in tumor cells for immune escape.

Discussion

Ablation of hematopoietic STAT3 results in antitumor immune responses in murine models of tumor cell transplantation.<sup>14</sup> These models have certain disadvantages since tumor cell lines have been cultured for a long period of time and may not ideally reflect tumor development in vivo. Our knowledge on the role of hematopoietic STAT3 in autochthonous tumors is limited. It is also unclear if and how tumors will adapt to a different microenvironment upon deletion of STAT3 to circumvent and escape the improved immune surveillance. Thus, we deleted STAT3 in myeloid cells in a murine model of autochthonous CRCs. The use of an autochthonous model allowed us to study expression data of the microdissected tumor cell tissue separately for processes involved in immunoediting.
cellular and molecular consequences of myeloid STAT3 ablation on the tumor stroma as well as on the tumor cells themselves. We found that ablation of myeloid STAT3 interfered with formation of inflammation-associated CRCs. Only few tumors evolved that seemed having adapted to escape the strong immunogenic microenvironment.

Inflammation is a strong promoter for CRC development but inflammatory infiltrates also contain immune cells with antitumor activities. Loss of STAT3 in hematopoietic cells provokes inflammation in the gut albeit it remained unclear whether this condition promotes or prevents formation of colitis-associated CRCs. Interestingly, we have not observed severe spontaneous colitis in STAT3mice. STAT3m and STAT3wt mice also responded to a comparable extent to DSS treatment regarding the activation status of intestinal inflammatory cells and ulceration score. Several reasons may underlie this discrepancy. It may be caused by the different C57BL/6 genetic background or alternatively may be related to a different intestinal microflora as STAT3m associated enterocolitis reacts to ablation of TLR4 signaling.

The increased angiogenesis observed in STAT3m CRCs remains controversial because it has been demonstrated that STAT3 activation in MDCs and TAMs of transplanted B16 melanomas favors tumor angiogenesis. However, the altered stromalization and chemokine/cytokine expression might have pleiotropic effects on angiogenesis pathways in STAT3m CRCs. The observed bias toward increased expression of positive regulators and decreased expression of negative regulators in the stroma of STAT3m tumors might result in a net increase of angiogenesis that cannot be attributed to a single factor. Interestingly, RORyt+ Th17 cells were almost completely absent in the stroma of STAT3m tumors. The role of this cell type in cancer is controversial and promoting as well as suppressive effects on angiogenesis have been reported. It has been demonstrated that inflammatory cytokines from local macrophages and dendritic cells can promote a Th17-Th1 “phenotypic drift” that leads to gradual acquisition of Th1-associated gene expression by Th17 cells. This is marked by loss of Th17 cell molecules including RORyt. It is possible that the STAT3-deficient myeloid cells promote a Th17-Th1 “phenotypic drift” thereby depleting the RORyt+ Th17 cell population in STAT3m tumors.

Upon challenge with transplanted tumor cells, the MC38 studies suggest that enhanced immune surveillance in STAT3m mice largely relies on myeloid cross activation of T cells. Most importantly, the stroma gene expression signatures in STAT3m tumors are clearly indicative for T cell activation whereas NK cell activities seem to play a minor role. In line with this hypothesis, CRC formation was not promoted in STAT3mNK mice which display reduced NK cell numbers and NK cell-dependent tumor surveillance including a significant reduction of Nkp46+ NK cells in the gut. The presence of CD3+CD8+, CD8+CD45RO+ stroma T cell infiltration and their intratumoral location in CRCs (Immunoscope) represents a better prognostic marker than conventional TNM staging. The additional determination of a core gene expression signature called ICR further refines the accuracy of prognosis. T cells were the most abundant stroma cell type in AOM/DSS-induced CRCs. Importantly, STAT3m tumors displayed upregulation of ICR genes irf1, stat1, cxcl9, and ccl5 indicating that they represent predictive markers in mice and humans.

The concept of immunoediting suggests that tumors – incompletely eradicated by the immune system – remain dormant and eventually escape immune attack (i.e. they become edited). CRCs in STAT3m mice face sustained stroma T cell activities that might force them to develop additional immune evasion mechanisms. The importance of STAT3 activation in tumor cells for immune evasion is well documented and has been demonstrated for various cancers including glioblastoma and squamous cell carcinoma. STAT3 is predominantly activated by IL-6 and IL-11 cytokines in CRCs. This cytokine-mediated STAT3 activation is usually transient but cancer cells have developed mechanisms for persistent activation of STAT3 that depends on sphingosine signaling. CRCs in STAT3m mice might represent a subset of survivors with particular high pY-STAT3 levels in tumor cells that selectively escaped sustained T cell activities. pY-STAT3 autoregulates its own expression leading to a positive feed forward loop that might explain the high levels of total STAT3 protein in STAT3m tumor cells.

STAT3 inhibitors are in clinical trials for various types of cancers and several immunotherapy strategies for CRC are currently tested. Our data suggest that application of STAT3 inhibitors interferes with immune evasion of CRC tumor cells which would improve the efficacy of immunotherapy strategies and prevent relapse.

Methods
Mice and in vivo experiments
C57BL/6 STAT3m (=LysMCre/Cre STAT3flox/flox) and STAT3wt control (=LysMCre/Cre STAT3wt/wt) mice were employed for tumor induction with AOM/DSS. For tumor transplantation studies, 1 × 106 of BCR/ABLp185 or 1 × 106 of MC38 cells were injected subcutaneously. Tumors were weighed and analyzed by flow cytometry 11 d (BCR/ABLp185) and 14 d (MC38) after transplantation. In vivo cytotoxicity of CTL cells was evaluated as previously described (supplemental methods). F4/80+ peritoneal macrophages were FACS sorted 4 h after i.p. injection of 4% thioglycoler (Sigma). All mouse experiments were performed in accordance with Austrian and European laws and with the general regulations specified by the Good Science Practices guidelines of the Medical University of Vienna.

In vitro cytotoxicity assay of NK cells
Mice were injected i.p. with PBS or 100 µg poly (I:C) to activate NK cells and spleen cells were isolated after 18 h. Splenocytes were incubated with 5 × 105 CFSE-stained (2.5 µM) YAC-1 target cells at effector-to-target ratios of 100:1, 50:1 and 25:1 in triplicates in 96-well plates. To assess the extent of spontaneously occurring apoptosis, tumor cells were incubated in the absence of NK cells. After 6 h 7-AAD Viability Solution was added and cytotoxicity was quantified by flow cytometry.
Cytotoxicity was normalized to quantified NK numbers of splenic single cell suspensions.

**Histology and immunohistochemistry (IHC)**

Intestines were flushed with PBS and 4% buffered formaldehyde and fixed as 4% formaldehyde. 4 μm paraffin sections were stained with H&E, alcin blue or IHC using standard procedures (supplemental methods).

**Flow cytometric analysis**

For staining of macrophages, splenic immune cells and lamina propria lymphocytes, cells were preincubated with PBS containing 0.5% BSA and FcyIII/IIIR antibody to block nonspecific binding. The Cell Trace CFSE Cell Proliferation Kit (Invitrogen) and the 7-AAD Viability Solution (eBioscience) was used according to the manufacturer’s instruction. Cells were stained with corresponding antibodies (supplemental methods) and analyzed using a FACSCanto II flow cytometer (BD Biosciences) or a FACS Fortessa (Beckton Dickinson) and data were calculated with FACSDiva software (BD Biosciences).

**Microarray analysis**

RNA from microdissected paraffin material was labeled and hybridized to mouse whole genome GeneChip Mouse Gene 2.0ST arrays (Affymetrix) according to manufacturer’s instructions. Microarray data were analyzed using Limma package of R with the parameters “mouse,” “issue type” (tumor and stroma) and “genotype” using a paired approach with “mouse” as pairing variable.55 p values were adjusted according to Benjamini-Hochberg.56 An adjusted p value < 0.05 was considered as significant. Data are available at GEO via accession number GSE60871.

**Weighted Gene Co-expression Network Analysis (WGCNA)**

The Pearson correlations were calculated for all pairs of genes in the samples. The resulting correlation matrix was transformed into a matrix of connection strengths by raising the absolute value of the correlation coefficients to the power β. A β of 6 was used to satisfy the scale free topology criterion.57 The top 25% of genes displaying the highest variability over the entire dataset were selected for module detection. From these genes, modules were determined using the “blockweise” module detection function of the WGCNA package with default values except for the minimal module size and the merge cut height parameters (set to 20 and 0.05, respectively). Biological functions of the single modules were determined using the GO term enrichment analysis function of WGCNA. Only “biological function” ontology was included into the analysis.58 p values of enriched terms were adjusted as above according to Benjamini-Hochberg. An adjusted p value < 0.05 was considered as significant.

**Statistics**

Significant differences in tumor parameters, IHC stainings and RT-PCR data were calculated with GraphPad Prism 5 software using unpaired t test and Mann–Whitney test. Multiple comparisons were calculated with One-way Anova and Bonferroni post-test. Significant differences between experimental groups were: *p < 0.05, **p < 0.01, or ***p < 0.005.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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