ANTIPROLIFERATIVE AGENT

Cross-reference to related application

[0001] This application claims benefit of, and priority from, U.S. provisional patent application No. 61/251,485, filed on 14 October 2009, the contents of which are hereby incorporated herein by reference.

Field of the Invention

[0001] The invention relates to antagonist of ANGPTL4 and methods of using the same including methods for the treatment of cancer and proliferative disorders.

Background

[0002] Cancer is one of the main diseases of the 21st century causing 13% of all deaths. While there are several chemical that can affect rapidly dividing cancer cells most of these are toxic with adverse side effects.

[0003] Cancer is expected to be the leading cause of death worldwide in the near future. Tumor cells exploit various signaling molecules to promote their growth, invasion and metastasis (1). A clear understanding of the mechanisms of actions of these molecules in malignancy will provide new insights into therapeutic interventions.

[0004] In response to stresses in the tumor microenvironment, such as hypoxia and inflammation, tumor cells exploit various signaling molecules to sustain and promote their growth, invasiveness and metastasis (Singh et al., 2007). Aggressive tumor metastasis and invasiveness is the main cause of mortality in cancer patients (Fidler, 1999). The constitutive activation of intracellular signaling by these molecules in tumor cells causes changes in cellular functions, including increased proliferation and the ability of cells to grow outside their original confined milieu, leading to metastasis (Pani et al., 2009; Westhoff and Fulda, 2009). Among these changes, the loss of dependence on integrin-mediated extracellular matrix contact for growth, or anoikis resistance, is an essential feature of tumor cells, yet how it is acquired remains an unsolved problem in cancer biology.

[0005] Although low levels of reactive oxygen species (ROS) regulate cellular signaling and play an important role in normal cell proliferation, recent studies show that tumors exhibit an excessive amount or persistent elevation of ROS (specifically the superoxide anion O_2^-) and utilize a redox-based mechanism to evade death by anoikis (Chiarugi, 2008; Giannoni et al., 2008; Giannoni et al., 2009; Pervaiz and Clement, 2007; Pervaiz and

Clément, 2002). Previous studies have indicated that ROS are involved in tumor initiation, progression and maintenance. Furthermore, deregulated ROS production is also associated with an invasive tumor phenotype. Oncogenic and mitogenic Ras activity is superoxide-dependent, and a sustained increase in ROS following the overexpression of Mox1 (the catalytic subunit of NADPH oxidase) leads to cell transformation and aggressive tumor metastasis (Komatsu et al., 2008; Suh et al., 1999). Elevated production of ROS following activation of the c-Met proto-oncogene leads to cell transformation and malignant growth (Ferraro et al., 2006), and Rac-dependent redox signals increase the secretion of metalloproteases and induce the epithelial-mesenchymal transition (Wu, 2006), which are two key features of invasive cancers. Thus, a clear understanding of the underlying redox-based anoikis escape mechanism and its connection to malignancy will provide new insights into therapeutic interventions.

[0006] Anoikis resistance, a hallmark of tumor malignancies (5, 6), is an integrin-dependent process (5). Reactive oxygen species (ROS) generated due to intergrin engagement oxidizes and activates Src, which stimulates the ERK and Akt pro-survival pathways (7, 8). Both pathways regulate the subcellular location or stability of BH3-only apoptotic proteins (eg Bad and Bim), essential for executing anoikis (10). Resistance to anoikis has been suggested to be a prerequisite for cancer cells to metastasize. The mechanism by which invading tumor cells survive the anoikis process remains largely unknown.

[0007] Angiopoietin-like protein 4 (ANGPTL4) are secreted proteins mainly expressed in liver that have been demonstrated to regulate triglyceride metabolism by inhibiting the lipolysis of triglyceride-rich lipoproteins (2). Experimental results show that ANGPTL4 function to regulate circulating triglyceride levels during different nutritional states and therefore play a role in lipid metabolism during feeding/fasting through differential inhibition of Lipoprotein lipase (LPL). The N-terminal domain of Angiopoietin-like proteins has been shown to play an active role in lipid metabolism. Using deletion mutants, it was demonstrated that the N-terminal domain containing fragment – (17–207) and not the C-terminal fibrinogen-like domain containing fragment – (207–460) increased the plasma triglyceride levels in mice. ANGPTL4 has been identified as a novel paracrine and, possibly, endocrine regulator of lipid metabolism (Oike et al., 2005) and a target of peroxisome proliferators-activated receptors (PPARs) (Kersten et al., 2000). It is expressed in numerous cell types, such as adipocytes and hepatocytes, and is upregulated after fasting and hypoxia (Belanger et al., 2002; Kersten et al., 2000). Importantly, ANGPTL4 undergoes proteolytic

processing to release its C-terminal fibrinogen-like domain (cANGPTL4), which circulates as a monomer yet whose function remains unclear. The N-terminal coiled-coil domain of ANGPTL4 (nANGPTL4) mediates the oligomerization of ANGPTL4 and binds to lipoprotein lipase to modulate lipoprotein metabolism (Ge et al., 2004). It is now established that the nANGPTL4 mediates its oligomerization and binds to lipoprotein lipase to modulate lipoprotein metabolism (Li, 2006; Sukonina et al., 2006). In contrast, cANGPTL4 exists as a monomer, and its function still remains unknown.

[0008] ANGPTL4 was recently linked to tumor progression. The angiopoietin-like 4 protein (ANGPTL4) has well-studied roles in metabolism, yet its role in cancer biology remains undefined. As a predictive gene for breast cancer metastasis (3), where it disrupts endothelial integrity (4). However, whether ANGPTL4 promotes or inhibits vascular permeability, and thus cancer metastasis remains controversial. There is apparently conflicting results as to the underlying mechanism of ANGPTL4 activity in tumor cells that have not been clarified, hampering our understanding of its precise role in cancer metastasis. The role of ANGPTL4 in cancer biology remains unesertianed.

Summary

[0009] The present invention seeks to ameliorate the above mentioned problems by providing composition such as an antibody or an SiRNA specific to angiopoietin like 4 protein (ANGPTL4) capable of neutralizing or knocking down the protein to halt or reduce cell proliferation and methods of making and using the same.

[0010] The invention provides an antagonist to angiopoietin like 4 protein (ANGPTL4) such as an antibody specific to angiopoietin like 4 protein (ANGPTL4) or an SiRNA capable of neutralizing or knocking down the protein to halt or reduce cell proliferation and methods of making and using the same.

[0011] The antagonist of the invention is further directed to the C terminal region of the protein and may be capable of neutralizing cell proliferation. The antagonist may be a monoclonal antibody and or a humanized antibody.

[0012] The present invention also provides a method of treating a patient to at least affect cell proliferation, which comprises the step of: contacting proliferating cell with an antagonist to angiopoietin like 4 protein (ANGPTL4). Preferably, the antagonist comprises (a) an antibody specific to angiopoietin like 4 protein (ANGPTL4) or (b) an antibody specific to the C terminal region of angiopoietin like 4 protein (ANGPTL4).

[0013] An alternative form of the present invention resides in the use of an antaogonist to angiopoietin like 4 protein (ANGPTL4) for the treatment of a tumor, Preferably, the antagonist comprises an antibody specific to angiopoietin like 4 protein (ANGPTL4) or an antibody specific to the C terminal region of angiopoietin like 4 protein (ANGPTL4) preferably the use at least affects growth of the tumor by either stopping the growth or reducing the size of the tumor.

[0014] The present invention also relates to compositions including pharmaceutical compositions comprising a therapeutically effective amount of an antagonist to angiopoietin like 4 protein (ANGPTL4). Preferably, the antagonist comprises (a) an antibody specific to or (b) an antibody specific to the C terminal region of . As used herein a compound will be therapeutically effective if it is able to affect cell proliferation.

[0015] The invention also provides a method of diagnosing proliferative disorders, comprising the steps of the determining an amount of the angiopoietin like 4 protein (ANGPTL4) protein in body fluids or tissue sampled from a person suspected of having a proliferative disorder.

[0016] Accordingly, the methods and compounds described herein may be used in diagnostic and therapeutic methods. Other aspects and advantages of the invention will become apparent to those skilled in the art from a review of the ensuing description, which proceeds with reference to the following illustrative drawings of preferred embodiments.

Brief Description of the Drawings

Figure 1. Suppression of ANGPTL4 impairs tumorigencity. Relative ANGPTL4 mRNA and protein levels in (a) HaCaT, HSC, II-4 and A-5RT3, (b) A-5RT3$_{ANGPTL4}$ and A-5RT3$_{CTRL}$ as evaluated by qPCR and immunoblotting. Only the C-teminal fibrinogen-like fragment of ANGPTL4 was detected. Values (mean±S.D.) from 5 independent analyses. (c) Tumour size induced by A-5RT3$_{ANGPTL4}$ compared to A-5RT3$_{CTRL}$at 8 weeks post injection. Each circle (mean±S.D.) represents 3 measurements from each mouse. (d) Immunodetection of proliferation (cyclin D1, PCNA), and apoptosis (cleaved capsase 3, PARP, Bax) markers in A-5RT3$_{ANGPTL4}$ and A-5RT3$_{CTRL}$ induced tumors. (e) Tumour size in mice injected *s.c.* with A-5RT3$_{CTRL}$ and treated *i.v.* with either mAb11F6C4 or control IgG. Values (mean±S.D.) from 5 mice. *:p<0.05, **:p<0.01. (f) Quantification of A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ tumour colonies on soft agar. Values (mean±S.D.) from four assays. (g) Apoptotic cells (%) after 2h anoikis was analyzed by FACS (events =5000) of three independent experiments.

Figure 2. ANGPTL4 modulates ROS generation via interactions with integrins. (a) Surface Plasmon resonance sensogram showing dose-dependent binding profiles of integrin β1 with immobilized-ANGPTL4 GLC chip. Sensorgram was corrected against a reference flow cell with no immobilized protein. K_D~10^{-7}M was determined after global fitting (Langmuir 1:1 model) using Scrubber2. *In situ* PLA detection of (b) ANGPTL4-integrin β1 complexes in indicated tumors; phosphorylated FAK and 14-3-3/Bad complexes in (c) A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ (d) tumors. PLA signals (red) and Hoechst-stained nuclei (blue); negative control without primary antibodies. Values (mean±S.D.) from 200 cells using BlobFinder (Uppsula University). Scale bars 40 μm. *:p<0.05. (e) Representative immunoblot of total or phosphorylated FAK. ERK and 14-3-3 from indicated tumors. Immunoprecipitation of reduced and activated c-Src in (f) A-5RT3$_{ANGPTL4}$ cells and (g) tumors. Cells were suspended for 1-2h (S1h, S2h). Cell lysates were labled with 100 μM N-(biotinoyl)-N'-(iodoacetyl) ethylenediamine to evaluate Src redox state.hHRP-Streptavidin immunoblot performed on anti-Src immunoprecipitates showed reduced Src. Immunoprecipitates were probed with anti-Src for normalization. Activated Src, Na^+ /H^- exchanger 1 and catalase were assessed using cognate antibodies. Representative pictures from three experiments. (h) Intracellular ROS of A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ evaluated by 5-(and 6-)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate acetyl ester. Data are normalized to total protein content. (i) Activities of caspases in A-5RT3$_{ANGPTL4}$ after 2 h suspension. . Values (mean±S.D.) represent a fold change above A-5RT3$_{CTRL}$ (n=3). *:p<0.05, **:p<0.01.

Figure 3 –Elevated ecpression of ANGPTL4 in tumors. Relative ANGPTL4 protein and mRNA levels in **(a)** paired human squamous cell carcinomas (SCC) and peri-tumor normal samples (PNSs) determined using immunoblotting and qPCR. Values (mean±S.D.) represent a fold change compared to HaCaT or cognate PNS samples. For paired human samples, each spot was the mean value of 3 different paraffin sections from an individual sample.Tissues from same individual are linked by coloured lines. Three SCCs with the highest ANGPTL4 corresponded to an invasive prognosis. (b) Expression of ANGPTL4 from laser capture microdisected (LCM) epithelial tumor and stromal fibroblasts from SSC and PNS. Hematoxylin and eosin images of SCC section, before and after LCM of epithelial tumor tissue. Microdissected tissues were processed for qPCR. Values (mean±S.D.) represent expression level from 5LCM pairs of PNS and SSC. (c) ANGPTL4 expression varies among solid tumors procured from different anatomic sites. Heatman profiles generated from immunofluorescence images using IMARIS (Bitplane Scientific Software). The X-Y axis

represents the length and width, while the Z axis represents the immunofluorescence intensity. Representative image of normal skin and skin tumor with its corresponding heatmap was shown. The heatmaps were grouped horizontally by respective anatomic sites.

Figure 4 – Suppression of ANGPTL4 in A-5RT3 cells impair tumorigenicity *in vivo*. **(a)** Lentivirus mediated suppression of ANGPTL4 has no off target effect. mRNA level of 2'5'-oligoadenylate synthetase isoforms 1 and 2 (OAS1, OAS2), interferon-induced myxovirus resistance 1 (MX1 and interferon-stimulated transcription factor 3γ (ISGF3γ) in A-5RT3$_{ANGPTL4}$ and A-5RT3$_{CTRL}$ determined by qPCR. Ribosomal protein L27 was used as a normalizing housekeeping gene. (b) Representative pictures of A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ induced tumors in two mice. Total of 5 mice per group. (c) Immunofluorescence staining of A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ induced tumors. Hematoxylin and eosin (H&E) stain of tumor sections. Proliferating (Ki67) and apoptotic (TUNEL-or cleved caspase 3) cells were identified using indicated antibodies or assay. Sections were counterstanined with DAPI (blue). Scale bars 40 μm. (d) Heatmap showing the genes up- and down regulated in A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ induced tumors as determined by qPCR. Results were generated from three pairs of indicated tumors. Detailed description of the genes and expression in fold changes is in Table 1. (e) ANGPTL4 interaction kinetic maps for mAbs, shown as association and dissociation rate constant (K_{on} and K_{off}) and a combination of K_{on} and K_{off} that results in the same affinity constant (K_D) values (diagonal lines) as determined by ProteOn XPR36 (Bio-Rad). Lables in maps identify the 6 mAb clones. mAb11F6C4 was chosen for subsequent immunotherapy experiment based on its superior K_{on}, K_{off} and K_D value. (f) Representative pictures of control IgG- or mAb 11F6C4-treated nude mice injected A-5RT3. Total of 6 mice per group. White arrows indicate injection site.

Figure 5 – ANGPTL4 interacts with integrins β5, but not β3, *in vitro* and *in vivo* (a) Sensorgram showed binding profiles between different concentrations of integrin β5 and immobilized ANGPTL4 chip. Each sensorgram was corrected as described in Figure 2a. Integrin β3 at 75 nM did not show any detectable interaction (dotted red). (b) Dose dependent ANGPTL4 binding to immobilized integrin αvβ5 (left panel) which was specifically blocked by anti-cANGPTL4 (right panel) as determined by ELISA. (c) Sensorgrams showed dose dependant blocking of integrin β1 (upper panel) and β5 (lower panel) to immobilized ANGPATL4 by pre-injection with different concentrations of mAb11F6C4. In situ PLA detection of ANGPTL4-integrin β1 and ANGPTL4-integrin β5

complexes in (d) A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ (e) A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ induced tumors, and of phosphorylated FAK and 14-3-3/Bad complexes in (f) A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$. Higher magnification images of 5e in left panel. PLA signals (red) and Hoechst-stained nuclei (blue). Negative control was without primary antibodies.

Figure 6 - Deficiency in ANGPTL4 induces apoptosis by anoikis. (a) Relative ANGPTL4 mRNA and protein levels in doxycycline-inducible suppression of ANGPTL4 in MDA-MB-231. A stable MDA-MB-231 cell line that expresses an anti-ANGPTL4 shRNA (Table 2) was produced using the Knockout Singe Vector System (Clontech). Cells were grown in the absence (-) or presence (+) of doxycycline (1 μg/ml) for 24h. +/- denates the removal of doxycycline after 24 h doxycycline treatment. Percentage of apoptotic cells of (b) MDA-MB-231 and (c) 11 different tumor cell lines after 2 h of forced suspension was evaluated by Annexin V-FITC/propidium iodide labling followed by FACS analysis (5000 events). MDA-MB-231 cells were treated with doxycycline for 24 h prior anoikis assay (right panel of (b)). Tumor cell lines were also exposed to mAb11F6C4 (10μg/ml). Values (mean ±S.D.) from three independent experiments. All examined tumor cell lines, the suppression of ANGPTL4 resulted in statistically more apoptotic cells, *:$p<0.05$ (student t-test).

Figure 7 – Deficiency in ANGPTL4 down regulates ROS production in tumor cells. ANGPTL4 level was suppressed either by inducible RNAi or by immunosuppression with mAb11F6C4 (10 μg/ml). Intracellular ROS production (in fluorescence units) was evaluated by means of CM-H_2DCFDA fluorescence dye. For MDA-MB-231, ROS was measured after 24 h treatment with 1 μg/ml doxycycline which suppressed endogenous ANGPTL4 by 85% (see Figure 4). Other cells were placed in suspension for 2h in the presence of increasing concentrations of either pre-immune IgG or Ab11F6C4, Values (mean ±S.D.) from three independent experiments. *:$p<0.05$, **:$p<0.01$.

Figure 8 – Deficiency in ANGPTL4 stimulates caspases 2, 3, 8 and 9 activities in tumor cell lines. Activities of caspases 2, 3, 6, 8, 9 were measured after 2 h of anoikis. Fold-increase in caspase activities was calculated by comparing with the caspase activities with pre-immune IgG or, for MDA-MB-231 in the absence of doxycycline. , Values (mean ±S.D.) from three independent experiments. *:$p<0.05$, **:$p<0.01$.

Figure 9 – The effect of the antibody on vascularisation A and B

Figure 10. Elevated Expression of ANGPTL4 in Various Tumor Types.

(A) ANGPTL4 expression varied among tumors procured from different anatomic sites. Heatmap profiles generated from immunofluorescence images using IMARIS (Bitplane Scientific Software). X and Y axes represent the length and width; Z axis represents immunofluorescence intensity. Representative images of normal skin and skin tumor samples with their corresponding heatmaps were shown. The heatmaps from same anatomic sites were grouped horizontally. Results are representative of two independent experiments with duplicates. Scale bars represent 200 μm. See Figure S1A-C.

(B) Relative ANGPTL4 mRNA and protein levels in non-tumorigenic skin line HaCaT and tumorigenic skin lines HSC, II-4, and A-5RT3. See Figure 18D.

(C-D) Relative ANGPTL4 mRNA and protein levels in paired human squamous cell carcinoma (SCC) (C) or basal cell carcinoma (BCC) (D) and cognate peri-tumor normal sample (PNS). Skin biopsies from normal human skin (NS) served as additional controls. Three SSCs with the highest mRNA ANGPTL4 levels corresponded with an invasive prognosis. See Figure 18E.

(E) Relative mRNA and protein levels of HIF1α in paired human SCC and PNS. For qPCR results, data spots from same individual were linked by coloured lines. See Figure 18F.

(B-E) mRNA data shown are mean ± SD from two independent qPCR experiments with triplicates. Ribosomal protein L27 (L27) was used as reference housekeeping gene. *$p < 0.05$; **$p<0.01$; ***$p<0.001$. Immunoblot data was from three independent experiments with duplicates. For immunoblot, β-tubulin served as loading and transfer control, and only cANGPTL4 was detected for immunoblot.

(F) Relative ANGPTL4 mRNA and protein levels in laser capture microdissected (LCM) epithelial cells and stromal fibroblasts from paired SCC and PNS. Hematoxylin and eosin images of SCC section, before and after LCM of epithelial tissue were shown in the left panel. Scale bars represent100 μm. Microdissected tissues were processed for qPCR (middle panel) and immunoblot (right panel) analyses.

Figure 11. Suppression of ANGPTL4 Impairs Tumorigenicity.

(A) Relative ANGPTL4 mRNA and protein levels in A-5RT3 (parental), A-5RT3$_{CTRL}$ (scrambled control) and A-5RT3$_{ANGPTL4}$ (knockdown) cells. Data are mean ± SD from three independent qPCR experiments with triplicates. Ribosomal protein L27 (L27) was used as reference housekeeping gene. Immunoblot data was from three independent experiments with duplicates. β-tubulin served as loading and transfer control. ***$p < 0.001$; n.s. denotes not significant.

(B) Size of xenograft tumors induced in nude mice by 5×10^5 of A-5RT3$_{ANGPTL4}$ or A-5RT3$_{CTRL}$ after 8 weeks post-inoculation (n = 5 each group). Each circle represents mean size from three measurements on each mouse at wk 8. ***$p < 0.001$. See Figure 19B.

(C) Representative pictures of A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$-induced tumors (wk 8) in (B). Black arrows indicate inoculation sites.

(D-E) Size of tumor volume induced in ANGPTL-knockout (KO) and wildtype (WT) mice (D), and PBS- or recombinant cANGPTL4-treated C57BL/6J WT mice (E) by B16F10 melanoma (B16F10$_{CTRL}$, control) and ANGPTL4-knockdown (B16F10$_{ANGPTL4}$). 1×10^6 indicated cells were *s.c.* inoculated for each mice. Mice were treated *i.v.* with either 3 mg/kg of cANGPTL4 or vehicle PBS thrice weekly (n = 6 each group) Values are mean ± SEM from three measurements on each mouse. *$p < 0.05$; **$p<0.01$; ***$p<0.001$). See Figure 19C-D.

(F) ANGPTL4 interaction kinetic maps for human monoclonal antibodies (mAbs), shown as association and dissociation rate constant (k_{on} and k_{off}), and a combination of k_{on} and k_{off} that results in the same affinity constant (K_D) values (diagonal lines) as determined by surface plasmon resonance (SPR). Labels in maps identify the 6 mAb clones. mAb11F6C4 was chosen for subsequent immunotherapy experiment based on its superior k_{on}, k_{off} and K_D value.

(G) Tumor volume in nude mice injected *s.c.* with 5×10^5 of A-5RT3 and treated *i.v.* with 30 mg/kg/week of either mAb11F6C4 or control IgG as a function of time (n = 6 for each group). Each circle represents mean ± SEM from three measurements on each mouse. *p < 0.05; **p<0.01; ***p<0.001.

(H) Representative pictures of control IgG- or mAb11F6C4-treated nude mice (wk 8) as described in (G). White arrows indicate A-5RT3 inoculation sites.

(I) Immunoblot of proliferation (PCNA and cyclin D1), and apoptosis (cleaved caspase-3, Bax and cleaved PARP) markers in A-5RT3$_{ANGPTL4}$- and A-5RT3$_{CTRL}$-induced tumor biopsies. Immunoblot data was from three independent experiments with duplicates. β-tubulin served as loading and transfer control.

(J) Hematoxylin and eosin (H&E) and inmunofluorescence staining of A-5RT3$_{CTRL}$- and A-5RT3$_{ANGPTL4}$-induced tumor sections. Proliferating (Ki67) and apoptotic (cleaved caspase-3 or TUNEL) cells were identified using indicated antibodies or assay. Sections were counterstained with DAPI (blue). Scale bars represent 40 μm.

(K) Heatmap showing genes up- and down-regulated in A-5RT3$_{ANGPTL4}$-induced tumors relative to A-5RT3$_{CTRL}$-induced tumors as determined by qPCR. Results were generated from three pairs of indicated tumors. Three independent qPCR experiments with triplicates were performed. Ribosomal protein L27 (L27) was used as reference housekeeping gene. Detailed description of the genes and expression see Table 1.

(I-K) All experiments were performed using tumor biopsies harvested from mice described in (B-C) at wk 8.

Figure 12. ANGPTL4 Interacts with Integrins β1 and β5 to Confer Tumor Cells Anoikis Resistance.

(A) Quantification of A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ tumor colonies on soft agar (left panel). Values (means ± SD) were obtained from four independent assays with triplicates. **$p < 0.01$. Representative pictures were shown in the right panel. Scale bars represent 1 mm.

(B) Percentage of apoptotic A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPT4}$ after 2 h of anoikis as analyzed by FACS (5000 events). The sum of Annexin V^+/PI^- (early apoptosis) and Annexin V^+/PI^+ (late apoptosis) cells were considered apoptotic. Values (bold) denote apoptotic cells (%). Results are representative of three independent experiments.

(C) Relative activities of caspases 2, 3, 6, 8, 9 in A-5RT3$_{ANGPTL4}$ compared with A-5RT3$_{CTRL}$ (assigned value 1) after 2 h of anoikis. Values (means ± SD) were obtained from three independent experiments with triplicates. *$p < 0.05$, **$p < 0.01$.

(D) Percentage of anoikis-induced apoptotic A-5RT3$_{ANGPT4}$ in the presence of increasing exogenous recombinant cANGPTL4 as analyzed by FACS (5000 events). Vehicle (PBS) treated A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPT4}$ served as controls for comparison. Apoptotic index as described in (B). See Figure 20A-C.

(E-F) Representative sensorgrams of three independent experiments showing binding profiles between immobilized-ANGPTL4 and integrin $\beta 1$ (E) and integrin $\beta 5$ (F). Integrin β3 at 75 nM did not show any detectable interaction (F, dotted red line). Sensorgram was corrected against a reference flow cell with no immobilized protein. $K_D \sim 10^{-7}$ M was determined after global fitting (Langmuir 1:1 model) using Scrubber2.

(G-H) Representative sensorgrams showed dose-dependent blocking of integrin β1 (G) and integrin β5 (H) to immobilized-ANGPTL4 by pre-injection with different indicated concentrations of mAb11F6C4. See Figure 20D-G.

(I-J) *In situ* proximity ligation (PLA) assay detection of ANGPTL4:integrin β1 (I, left two panels), ANGPTL4:integrin β5 (I, right two panels), and phosphorylated FAK (J) in A-

$5RT3_{ANGPTL4}$- and A-$5RT3_{CTRL}$-induced tumor biopsies. Higher magnification images are shown in (H, 2nd and 4th panels; J, right panel). PLA signals are shown in red and nuclei are stained blue by Hoechst dye. Images were acquired in one z-plane using a Zeiss LSM710 META confocal laser scanning microscope. Negative controls were performed with only anti-nANGPTL4 (I) or anti-FAK (J) antibodies. Scale bars represent 40 μm.

(K) Immunoprecipitation and immunodetection of ANGPTL4, integrin β1, integrin β5, total FAK, phosphorylated FAK (pY397FAK), total Rac1 and GTP-bound Rac1 (GTP-Rac1), from indicated tumor sections. Configuration-specific monoclonal anti-Rac-GTP antibody (NewEast Biosciences) was using for immunoprecipitation GTP-Rac1. Total FAK served as loading and transfer control. Experiments in (I-K) were performed using tumor biopsies harvested from A-$5RT3_{CTRL}$- or A-$5RT3_{ANGPTL4}$-inoculated (5×10^5 cells each) nude mice at wk 8 (Figure 11B-C). See Figure 20H-J. All experiments in (B-K) were repeated for at least three times with consistent results.

Figure 13. ANGPTL4 Elevates O_2^- Level and Maintains Relatively High O_2^-:H_2O_2 Ratio in Tumor Cells.

(A, E and G) Representative electron paramagnetic resonance (EPR) spectra of DEPMPO-superoxide spin adduct from A-$5RT3_{CTRL}$ and A-$5RT3_{ANGPTL4}$ (A), A-$5RT3_{CTRL}$- and A-$5RT3_{ANGPTL4}$-induced tumor (E) or MDA-MB-231 (G) in the absence or presence of indicated chemicals or inhibitors. MDA-MB-231 cells were treated with mAb11F6C4 (3 or 6 μg/ml) or control IgG (6 μg/ml). A-$5RT3_{CTRL}$ and A-$5RT3_{ANGPTL4}$ were transiently transfected with vector expressing Rac1(T17N) or Rac1(G12V), respectively. A-$5RT3_{CTRL}$, A-$5RT3_{ANGPTL4}$ and MDA-MB-231 were transiently transfected with ON-TARGETplus siRNA (Dharmacon) against either Nox1 (Nox1 kd) or Nox2 (Nox2 kd). Superoxide adduct of DEPMPO has a hyperfine splitting constants of a_N=13.13 G; a_P=55.61 G; a^{β}_H=13.11 G; a^{γ}_H=0.71, 0.42, 0.7, 0.25, and 0.6 G. See Figure 21.

(B, F and H) EPR signal intensity at 3480 G from A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ in (A), A-5RT3$_{CTRL}$- and A-5RT3$_{ANGPTL4}$-induced tumor in (E) or MDA-MB-231 in (G). Tiron treated measurement served as negative signal control.

(C and I) Measurement of O_2^- levels using MCLA assay in A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ (C), or MDA-MB-231 treated with mAb11F6C4 (3 or 6 μg/ml) or control IgG (6 μg/ml) (I) in the absence or presence of indicated chemicals or inhibitors.

(D and J) Measurement of H_2O_2 levels using Amplex red assay in A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ (D), or in MDA-MB-231 treated with mAb11F6C4 (3 or 6 μg/ml) or control IgG (6 μg/ml) (J). Arbitrary relative O_2^-:H_2O_2 ratios were shown in boxes. See Figured 21D and S4F.

(B-D and F-J) Values were normalized to total proteins and were presented as mean ± SEM as were obtained from three independent experiments with triplicates. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; n.s. represents not significant. Vehicle-treated A-5RT3$_{CTRL}$ (B and C), A-5RT3$_{CTRL}$-induced tumor (F) and MDA-MB-231 in the presence of control IgG (6 μg/ml) (H and I) served as cognate controls.

(K) Percentage of apoptotic MDA-MB-231 after 2 h of anoikis as analyzed by FACS (5000 events). Apoptotic index as described in (Figure 12B). Values (bold) denote apoptotic cells (%) from three independent experiments.

(L) Relative activities of caspases 2, 3, 6, 8, 9 in mAb11F6C4-treated MDA-MB-231 after 2 h of anoikis. Values (means ± SD) were from three independent experiments with triplicates. *$p < 0.05$; **$p < 0.01$. Fold-increase in caspase activities was calculated by comparing with pre-immune IgG-treated MDA-MB-231.

Figure 14. ANGPTL4-mediated O_2^- Regulates Src and Promotes PI3K/PKBα and ERK Survival Pathways.

(A and D) Immunoblot of indicated proteins in A-5RT3$_{ANGPTL4}$- and A-5RT3$_{CTRL}$-induced tumor biopsies. Values represent mean fold change from four independent experiments. c-Src (A) and β-tubulin (D) served as respective loading and transfer control.

(B) Immunoblot of indicated proteins in A-5RT3$_{ANGPTL4}$ and A-5RT3$_{CTRL}$ in the absence or presence of 20 μM diphenylene iodonium (DPI), and in Nox1-knockdown (Nox1 kd) A-5RT3$_{ANGPTL4}$ and A-5RT3$_{CTRL}$. Cells were suspended for 0, 1 and 2 h (S0h, S1h and S2h). Cell lysates were labelled with 100 μM *N*-(biotinoyl)-*N'*-(iodoacetyl) ethylenediamine to evaluate Src redox state. HRP-Streptavidin immunoblot performed on anti-Src immunoprecipitate showed reduced Src. Immunoprecipitate was probed with anti-c-Src for normalization. Values (mean ± SD) represent mean fold change against value at S0h. Data shown are representatives of three independent experiments.

(C) Percentage of apoptotic A-5RT3$_{ANGPTL4}$ and A-5RT3$_{CTRL}$ cells, treated with either MEK inhibitor PD98059 or PI3K inhibitors LY294002 and Wortmannin, after 2 h of anoikis challenge and analyzed by FACS (5000 events). The sum of Annexin V^+/PI^- and Annexin V^+/PI^+ cells were considered apoptotic. Values were obtained from three independent experiments.

(E) *In situ* PLA detection of 14-3-3:Bad complexes in indicated tumor sections and cells. PLA signals were red dots and Hoechst stained nuclei blue. The cells were counterstained with Alexa488-phallodin for actin stress fibers (green). Negative controls were performed with only anti-14-3-3 antibodies. Images were acquired in one z-plane using a Zeiss LSM710 META confocal laser scanning microscope. Data shown are representative of three independent experiments. Scale bars represent 40 μm.

(F) Mean number of 14-3-3:Bad complexes (as shown in E, right panel) was calculated from 200 cells (n = 3; total 600 cells) using BlobFinder software. Error bars represent SD. ***$p < 0.001$.

Figure 15. ANGPTL4 Maintains Relatively High O_2^-:H_2O_2 Ratio In Tumor Cells.

Measurement of O_2^- (A) and H_2O_2 (B) levels in nine different tumor lines. O_2^- was determined using a chemiluminescence MCLA assay. The level of H_2O_2 was determined using Amplex red assay, in the presence of specific catalase inhibitor, 3-amino-1, 2, 4-triazole. Arbitrary relative O_2^-:H_2O_2 ratios (B) were shown in boxes. Values (mean ± SD) were normalized to total protein content. Three independent experiments were performed with consistent results. *$p < 0.05$; **$p < 0.01$.

Figure 16. Deficiency of ANGPTL4 Activates Caspase Activities and Induces Apoptosis Upon Anoikis in Tumor Cells.

(A) Relative activities of caspases 2, 3, 6, 8, 9 were measured after 2 h of anoikis. Fold-increase of caspase activities in mAb11F6C4 (6 μg/ml)-treated cells was calculated by comparing with the caspase activities treated with pre-immune IgG (6 μg/ml). Values (mean ± SD) were obtained from at least three independent experiments with consistent results.*$p < 0.05$; **$p < 0.01$.

(B) Percentage of apoptotic cells of 9 different tumor cell lines after 2 h of anoikis was evaluated by Annexin V-FITC/propidium iodide labelling followed by FACS analysis (5000 events). Tumor cells were treated with 10 μg/ml of control IgG or mAb11F6C4. Apoptotic index was as described in legend of Figure 12B. Results are representative of at least three independent experiments. $p < 0.05$.

(C) Relative ANGPTL4 mRNA (left panel) and protein (middle panel) levels in MDA-MB-231, whose ANGPTL4 suppression were doxycycline-inducible. A stable MDA-MB-231 cell line that expresses an anti-ANGPTL4 shRNA (see supplemental experimental procedures) was produced using the Knockout Singe Vector System (Clontech). Cells were grown in the absence (-) or presence (+) of doxycycline (1 μg/ml) for 24 h. +/- denotes the removal of

doxycycline after 24 h doxycycline treatment. The right panel shows percentage of apoptotic cells of MDA-MB-231 as evaluated by anoikis assay. Data shown were obtained from three independent experiments with consistent results.

Figure 17. ANGPTL4-mediated Regulation of O_2^- Production in Tumor.

In an autocrine manner, tumor-derived ANGPTL4 specifically bind to integrins β1 or β5 and subsequently activates the FAK and Rac1 activities, which further activates the NADPH oxidase-dependent generation of "*onco-ROS*" O_2^-, promoting a relatively high O_2^-:H_2O_2 ratio in tumor cells. This pro-oxidant intracellular milieu, which may subsidiarily maintained through NHE, favours cell survival and proliferation by oxidizing/activating the Src machinery and therefore stimulates its downstream PI3K/PKBα- and ERK-mediated survival pathways. This further triggers the 14-3-3 adaptor protein to sequester the pro-apoptotic Bad from mitochondria to prevent apoptosis and favour cell survival.

Figure 18. Elevated Expression of C-terminal ANGPTL4 (cANGPTL4) in Tumors, related to Figure 10.

(A and B) Hematoxylin and eosin (H&E) image (A) and immunofluorescence image probed with anti-cANGPTL4 antibody (B) on melanoma tumor tissue (representative of the tumor biopsies from array shown in Figure 10A). Higher magnification pictures randomly selected from the melanoma tissue were shown on (A, right panel) and (B, DAPI on the middle and cANGPTL4 on the right panel), respectively. The heatmap (B, left bottom panel) was transformed from the immunofluorescence image (B, right upper panel) based on the gray value (immunofluorescence intensity) of cANGPTL4 as described in Figure 10A. Scale bars represent 200 μm.

(C) Average integrated gray value (immunofluorescence intensity) of cANGPTL4 from various normal and tumor tissues (also see Figure 10A). Tissues from same anatomic site

were grouped and compared. Values (mean ± SEM) were calculated from at least three biopsies and microscopic fields of each tissue. *$p < 0.05$; **$p < 0.01$.

(D-E) Immunoblot analysis using anti-nANGPTL4 antibody of tumorigenic skin lines HSC, II-4, and A-5RT3 (D), and human skin squamous cell carcinomas (SCCs), basal cell carcinomas (BCCs) and cognate peri-tumor normal sample (PNS) (E). Liver, non-tumorigenic skin line HaCaT and normal skin biopsies (NS) served as cognate positive controls. Coomassie stained blot or β-tubulin served as loading and transfer control. No full-length or nANGPTL4 was detected in indicated tumor cell line, BCCs or SCCs. Anti-nANGPTL4 antibody was previously described (Kersten et al., 2000).

(F) HIF1α along with ANGPTL4 mRNA levels were concomitantly up-regulated in SSCs when compared with PNSs with a Pearson correlation coefficient of 0.88. See the individual mRNA expression of ANGPTL4 and HIF1α in SCCs in Figures 10C and 10E.

(G-I) Relative mRNA expressions of peroxisome proliferator-activated receptor α (PPARα) (G), PPARδ (H) and PPARγ (I) in paired human squamous cell carcinomas (SCCs) and peri-tumor normal samples (PNSs) as determined by qPCR. Data spots from same individual were linked by coloured lines. Data shown are mean ± SD from two independent qPCR experiments with triplicates. Ribosomal protein L27 (L27) was used as reference housekeeping gene. n.s. represents not significant in the comparison between paird SCCs and PNSs.

Figure 19. Suppression of ANGPTL4 Reduces Tumorigenicity and Exogenously Infused cANGPTL4 Accelerates Tumor Growth, related to Figure 11.

(A) Relative mRNA levels of key interferon response genes: 2',5'-oligoadenylate synthetase isoforms 1 and 2 (Oas1, Oas2), interferon-induced myxovirus resistance 1 (Mx1) and interferon-stimulated transcription factor 3γ (Isgf3γ) in A-5RT3 (parental cell), A-5RT3$_{CTRL}$ (scrambled control cell) and A-5RT3$_{ANGPTL4}$ (ANGPTL4 knockdown cell). Results shown

are mean ± SD from three independent qPCR experiments with triplicates. Ribosomal protein L27 (L27) was used as reference housekeeping gene. n.s. represents not significant in the comparisons between A-5RT3 and A-5RT3$_{ANGPTL4}$ or between A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$.

(B) Mean zize of xenograft tumors induced in nude mice by 0.5x, 2x and 8x10^6 A-5RT3$_{ANGPTL4}$ or A-5RT3$_{CTRL}$ after 4 weeks post-inoculation (each group). Values (mean ± SEM) were calculated from n = 5 (each group) mice. *$p < 0.05$; ***$p<0.001$

(C) Representative pictures of B16F10-induced tumors in C57BL/6J mice with *i.v.* treatments of either 3mg/kg of cANGPTL4 or control PBS thrice a week and dissected 15 days after injection (scale bar 10mm). See also Figure 11E.

(D) Immunoblot detection of recombinant cANGPTL4 using anti-His-tag and anti-cANGTPL4 antibodies. Plasma samples from C57BL/6J mice 1 day post-treatment with cANGPTL4 or control PBS (as described in Figure 11E) were used. Coomassie stained blot served as loading and transfer control. Experiments were repeated three times with consistent results.

Figure 20. ANGPTL4 Effects on Keratinocytes and Its Interaction With Integrins to Activate FAK, related to Figure 12.

(A) Percentage of anoikis-induced apoptotic skin keratinocytes and ANGPTL4-deficient keratinocytes in the presence of increasing exogenous recombinant cANGPTL4 as analysed by FACS (5000 events). Vehicle(PBS)-treated keratinocytes and ANGPTL4-deficient keratinocytes served as cognate controls for comparison. Apoptotic index as described in (Figure 12B).

(B-C) Apoptotic index of attached epithelial cells. A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPT4}$ (B), and skin keratinocytes and ANGPTL4-deficient keratinocytes (C) were detached by trypsin, subjected for Annexin V and PI staining, and immediately analysed by FACS (5000 events).

The sum of Annexin V^+/PI^- and Annexin V^+/PI^+ cells were considered dead. Values (bold) denote death rate (%).

(D-G) Dose-dependent ANGPTL4 bindings to immobilized integrin αvβ5 (D and E) and integrin α5β1 (F and G), which were specifically blocked by anti-cANGPTL4 as determined by ELISA.

(H) Immunoblot detects no significant difference in the protein expressions of integrins β1, β5 and β3 between A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ cells.

(I-J) *In situ* PLA detection of ANGPTL4:integrin β1 and ANGPTL4:integrin β5 complexes (I), and of phosphorylated FAK (J) in A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ cells. PLA signals are shown in red and nuclei were stained blue by Hoechst dye. The cells were also counterstained with Alexa488-phallodin for actin stress fibers (green). Negative controls were performed with only anti-nANGPTL4 (I) or anti-FAK (J) antibodies. Images were acquired in one z-plane using a Zeiss LSM710 META confocal laser scanning microscope. Scale bars represent 40 μm. PLA images are representative of three independent experiments. Graph (J, right panel) showed mean number of phosphorylated FAK calculated from 200 A-5RT3$_{ANGPTL4}$ and A-5RT3$_{CTRL}$ cells (n = 3; total 600 cells) using BlobFinder software (Uppsula University). Error bars represent SD. *$p < 0.05$. All experiments were performed three or four times with consistent results.

Figure 21. Suppression of ANGPTL4, Nox1 and Nox2 in Tumor Cells, related to Figure 13.

(A) Suppression of ANGPTL4 has no effect in the methionine/homocysteine metabolic cycle of tumor cells Relative mRNA level of Bhmt, Mat1a, Ahcy, Khk, Oat and Hacl1 (representative genes in the methionine/homocysteine metabolic cycle) in A-5RT3$_{ANGPTL4}$ and A-5RT3$_{CTRL}$ as determined by qPCR.

(B) Immunoblot of Nox1 and Nox2 in A-5RT3$_{CTRL}$, A-5RT3$_{ANGPTL4}$ and MA-MB-231 cells. β-tubulin served as loading and transfer control. Representative blots of three independent experiments were shown.

(C and E) Relative Nox1 and Nox2 mRNA and protein levels in A-5RT3$_{CTRL}$ (scrambled control), A-5RT3$_{Nox1}$ (Nox1 knockdown) and A-5RT3$_{Nox2}$ (Nox2 knockdown) cells (C), and in MDA-MB-231$_{CTRL}$ (scrambled control), MDA-MB-231$_{Nox1}$ (Nox1 knockdown) and MDA-MB-231$_{Nox2}$ (Nox2 knockdown) cells (E).

(D and F) Measurement of H_2O_2 levels using Amplex red assay in A-5RT3$_{CTRL}$ and A-5RT3$_{Nox1}$ (D), and in MDA-MB-231$_{CTRL}$ and MDA-MB-231$_{Nox1}$ cells (F).

(A and C-E) Error bars represent SD from three independent qPCR experiments with triplicates. Ribosomal protein L27 (L27) was used as reference housekeeping gene. ***$p < 0.001$; n.s. represents not significant.

Detailed Disclosure

[0017] We examined the expression pattern of ANGPTL4 in various types of human tumor cells and found that an elevated level of ANGPTL4 is a common feature of many tumor types. Next, we found that suppression of ANGPTL4, either by RNA interference or monoclonal antibody, significantly impaired the growth of tumor cells *in vivo* and their resistance to anoikis *in vitro*. Further study of the underlying mechanism of ANGPTL4 activity led us to propose that tumor cells express ANGPTL4 to modulate intracellular O_2^- levels, conferring anoikis resistance to tumor cells and promoting tumorigenesis and that, therefore, ANGPTL4 is a potentia

[0018] We showed that only cANGPTL4 was detected and elevated in many human tumor cells, predominantly secreted by the proliferative tumor epithelial cells. cANGPTL4 specifically binds to integrins $\beta 1$ and $\beta 5$ on tumor cells and activates the FAK and Rac1, which further stimulates NADPH oxidase-mediated O_2^- production by an autocrine pathway. However, it is conceivable that in tissues/organs expressing high level of cANGPTL4 in proximity to the tumor site may trasmit a paracrine signal.l therapeutic target in cancer treatment.

[0019] Consistent with the invention there is provided an antaginost to the angiopoietin like 4 protein (ANGPTL4) such as (a) an antibody specific to the angiopoietin like 4 protein (ANGPTL4) and, or (b) an antibody specific to the C terminal of the protein or (c) SiRNA. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

[0020] Antibodies directed to the C terminus of ANGPTL4 demonstrated superior association, dissociation, and affinity constant. C-teminal expesses fibrinogen-like fragment of ANGPTL4.

[0021] Here, we found that elevated ANGPTL4 expression is widespread in tumors, and its suppression impairs tumor growth associated with enhanced apoptosis. Tumor-derived ANGPTL4 interacts with integrins to stimulate the NADPH oxidase-dependent production of O_2^- A high ratio of O_2^-:H_2O_2 oxidizes/activates Src, triggering the PI3K/PKBα and ERK pro-survival pathways to confer anoikis resistance, thus promoting tumor growth. ANGPTL4 deficiency results in diminished O_2^- production and a reduced O_2^-:H_2O_2 ratio, creating a cellular environment conducive to apoptosis. Thus, ANGPTL4 is a novel redox factor in cancer biology and a potential therapeutic target.

[0022] The following embodiments are encompassed by the present invention:

[0023] An immunoglobulin that specifically binds to the C terminal of the ANGPTL4 protein.

[0024] The immunoglobulin wherein said immunoglobulin comprises an immunoglobulin heavy chain.

[0025] The immunoglobulin comprising an immunoglobulin light chain.

[0026] The immunoglobulin wherein said immunoglobulin is an IgG1 kappa immunoglobulin.

[0027] The immunoglobulin wherein said immunoglobulin comprises a human IgG1 constant region within a heavy chain of said immunoglobulin and a human constant region within a light chain of said immunoglobulin.

[0028] The immunoglobulin wherein said immunoglobulin comprises fully or partially human framework regions within the variable domain of said heavy chain and within the variable domain of said light chain.

[0029] The immunoglobulin wherein said immunoglobulin comprises murine framework regions within the variable domain of said heavy chain and within said light chain.

[0030] The immunoglobulin wherein said immunoglobulin is conjugated to an agent selected from the group consisting of a therapeutic agent, a prodrug, a peptide, a protein, an enzyme, a virus, a lipid, a biological response modifier, a pharmaceutical agent , and PEG.

[0031] A composition comprising the immunoglobulin of the invention and a carrier.

[0032] A method for preventing or treating proliferative disorders in a subject comprising administering to said subject an effective amount of a composition comprising the immunoglobulin of the invention.

[0033] A pharmaceutical composition comprising the immunoglobulin of the invention.

[0034] A method of diagnosing a proliferative disorders, comprising the steps of (a) determining an amount of the angiopoietin like 4 protein (ANGPTL4) protein in body fluids or tissue sampled from a person suspected of having a proliferative disorder (b) comparing the amount of the angiopoietin like 4 protein (ANGPTL4) from a person suspected of having a proliferative disorder with a second amount sampled from a healthy individual wherein elevated ANGPLT4 in the first sample compared with the second sample is indicative that a proliferative disorder is present in the person suspected of having a proliferative disorder.

[0035] A method of diagnosing proliferative disorders in a subject comprising, removing a sample from the subject, contacting the sample with a composition of the invention and detecting the presence of ANGPTL4 wherein elevated ANGPLT4 is a sample compared with a standard of normal ANGPTL4 expression is indicative that a proliferative disorder is present in the subject.

Polyclonal Antibodies

[0036] The antibodies of the invention may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant.

[0037] Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The intensity of the response is determined by several factors including the size of the immunogen molecule, its chemical characteristics, and how different it is from the animal's own proteins. Most natural immunogens are proteins with a molecular weight above 5 kDa that come from sources phylogenically far removed from the host animal (i.e., human proteins injected into rabbits or goats). It is desirable to use highly purified proteins as immunogens, since the animal will

produce antibodies to even small amounts of impurities present as well as to the major component. The antibody response increases with repeated exposure to the immunogen, so a series of injections at regular intervals is needed to achieve both high levels of antibody production and antibodies of high affinity.

[0038] To the extent that the antagonist is an antibody that engage the c terminal of the fibrinogen-like fragment of ANGPTL4 preventing cell proliferation, the immunogen will be an selected from amino acids comprising the c terminal of the fibrinogen-like fragment of ANGPTL4. Preferably, the amino acid sequence will be selected from the region of about 186 - 406 in the human ANGPTL4 protein or about 190 - 410 in the mouse ANGPTL4 protein. Sequences of at least 5, 6, 7, 8, 9, 10, 15, 20, 25, 30 amino acids from this region will generally be used to generate those antibodies. Desirably, the sequence selected will generate an antibody that specifically interferes with binding of ANGPTL4 to apoptosis inducing compounds .

[0039] Not all immunogenic molecules will however generate the level of antibody desired. To increase the intensity of the immune response immunogens are combined with complex mixtures called adjuvants. Adjuvants are a mixture of natural or synthetic compounds that, when administered with antigens, enhance the immune response. Adjuvants are used to (1) stimulate an immune response to an antigen that is not inherently immunogenic, (2) increase the intensity of the immune response, (3) preferentially stimulate either a cellular or a humoral response (i.e., protection from disease versus antibody production). Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). A more extensive discussion of adjuvants and their use in immunization protocols is given in Immunology Methods Manual, vol. 2, I. Lefkovits, ed., Academic Press, San Diego, CA, 1997, ch. 13. Immunology Methods Manual is available as a four volume set, (Product Code Z37,435-0); on CD-ROM, (Product Code Z37,436-9); or both, (Product Code Z37,437-7)

[0040] If the immunogen is still unable to generate an acceptable response, it may be conjugated to a carrier protein that is more immunogenic. Small molecules such as drugs, organic compounds, and peptides and oligosaccharides with a molecular weight of less than 2-5 kDa like, for example, small segments if ANGPTL4 such as those with a fibrinogen-like fragment in their structure, may not be immunogenic, even when administered in the presence of adjuvant. In order to generate an immune response to these compounds, it is necessary to attach them to a protein or other compound, termed a carrier that is

immunogenic. When attached to a carrier protein the small molecule immunogen is called a hapten. Haptens are also conjugated to carrier proteins for use in immunoassays. The carrier protein provides a means of attaching the hapten to a solid support such as a microtiter plate or nitrocellulose membrane. When attached to agarose they may be used for purification of the anti-hapten antibodies. They may also be used to create a multivalent antigen that will be able to form large antigen-antibody complexes. When choosing carrier proteins, remember that the animal will form antibodies to the carrier protein as well as to the attached hapten. It is therefore relevant to select a carrier protein for immunization that is unrelated to proteins that may be found in the assay sample. If haptens are being conjugated for both immunization and assay, the two carrier proteins should be as different as possible. This allows the antiserum to be used without having to isolate the anti-hapten antibodies from the anti-carrier antibodies.

[0041] Where the immunizing agent is a fibrinogen-like fragment segment such as from the c terminal preferably the fibrinogen-like fragment segment is conjugated to a protein known to be immunogenic in the mammal being immunized.

[0042] Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin (KLH), serum albumin, bovine thyroglobulin, soybean trypsin inhibitor, and a toxoid, for example tetanus toxoid.

[0043] KLH is a respiratory protein found in molluscs. Its large size makes it very immunogenic, and the large number of lysine residues available for conjugation make it very useful as a carrier for haptens. The phylogenic separation between mammals and molluscs increases the immunogenicity and reduces the risk of cross-reactivity between antibodies against the KLH carrier and naturally occurring proteins in mammalian samples.

[0044] KLH is offered both in its native form, for conjugation via amines, and succinylated, for conjugation via carboxyl groups. Succinylated KLH may be conjugated to a hapten containing amine groups (such as a peptide) via cross-linking with carbodiimide between the newly introduced carboxyl groups of KLH and the amine groups of the hapten.

[0045] Protocols for conjugation of haptens to carrier proteins may be found in *Antibodies: A Laboratory Manual,* E. Harlow and D. Lane, ed., Cold Spring Harbor Laboratory (Cold Spring Harbor, NY, 1988) pp. 78-87 (Product Code A 2926)

[0046] The immunization protocol may be selected by one skilled in the art without undue experimentation. Protocols for preparing immunogens, immunization of animals, and collection of antiserum may be found in *Antibodies: A Laboratory Manual,* E. Harlow and D.

Lane, ed., Cold Spring Harbor Laboratory (Cold Spring Harbor, NY, 1988) pp. 55-120 (Product Code A 2926).

Monoclonal Antibodies

[0047] The antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein (1975), Nature, 256:495. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent as described above to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

[0048] Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

[0049] Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, Calif. and the American Type Culture Collection, Manassas, Va. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. (1984) Immunol., 133:3001).

[0050] The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against ANGPTL4 and/or the the c terminal of ANGPTL4 or any of the sequences SEQ ID No. 1 to 3.

[0051] After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

[0052] The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

[0053] The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Pat. No. 4,816,567.

[0054] The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain cross-linking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent cross-linking.

[0055] *In vitro* methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art.

Human and Humanized Antibodies

[0056] The antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding sub-sequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR

regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

[0057] Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones *et al.*, (1986) Nature, 321:522-525; Riechmann *et al.*, (1988) Nature, 332:323-327; Verhoeyen *et al.*, (1988) Science 239:1534-1536], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

[0058] Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. (1991) Mol. Biol., 227:381; Marks et al., (1991) J. Mol. Biol., 222:581]. The techniques of Cole *et al.* and Boerner *et al.* are also available for the preparation of human monoclonal antibodies (Cole *et al.*, (1985) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 and Boemer *et al.*, (1991) J. Immunol., 147(1):86-95]. Similarly, human antibodies can be made by introducing of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks *et al.*, (1992) Bio/Technology 10, 779-783; Lonberg *et al.*, (1994) Nature 368 856-859; Morrison, (1994) Nature 368, 812-13; Fishwild *et al.*, (1996) Nature Biotechnology 14, 845-51; Neuberger, (1996) Nature Biotechnology 14, 826; Lonberg and Huszar, (1995) Intern. Rev. Immunol. 13 65-93.

Bispecific Antibodies

[0059] Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for ANGPTL4 and/or a segment of ANGPTL4 comprising portions of the c terminal of the fibrinogen-like fragment of ANGPTL4, the other one is for another compound having ANGPTL4.

[0060] Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Milstein and Cuello, (1983) Nature, 305:537-539].

Heteroconjugate Antibodies

[0061] Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [U.S. Pat. No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

Immunoconjugates

[0062] The invention also pertains to immunoconjugates comprising an antibody conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g., an enzymatically active toxin against hemagglutinin), or a radioactive isotope (i.e., a radioconjugate).

[0063] Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein-coupling agents such as N-succinnimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutareldehyde), bis-azido compounds (such as bis(p-azidobenzoyl)hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene).

Method of treatment and or use of the antibodies of the invention

[0064] The present invention also provides a method of treating a patient to at least affect a proliferative disorder, which comprises the step of: contacting a cell with an antagonist such as (a) an antibody specific to angiopoietin like 4 protein (ANGPTL4) or (b) an antibody specific to the C terminal region of angiopoietin like 4 protein (ANGPTL4). Preferably, the antagonist interferes with cell proliferation by means that neutralize angiopoietin like 4 protein (ANGPTL4) expression.

[0065] An alternative form of the present invention resides in the use of an antibody specific angiopoietin like 4 protein (ANGPTL4) or an antibody specific to the C terminal region of angiopoietin like 4 protein (ANGPTL4) for the treatment of cancer, preferably the use at least affects cell proliferation.

[0066] Cancer may include, all types of known tumors that exhibit over expression of angiopoietin like 4 protein (ANGPTL4). Cell proliferating or tumor refers to cells that are growing uncontrollably.

[0067] "Treatment" and "treat" and synonyms thereof refer to therapeutic treatment wherein the object is to prevent or slow down (lessen) a tumor. Treatment may include prophylactic passive immunization or immunotherapy treatment of a patent. Those in need of such treatment include those with a proliferative disorder.

[0068] As used herein a "therapeutically effective amount" of a compound will be an amount of active agent that is capable of preventing or at least slowing down (lessening) cell proliferation or tumerogenesis. Dosages and administration of an antagonist of the invention in a pharmaceutical composition may be determined by one of ordinary skill in the art of clinical pharmacology or pharmacokinetics. See, for example, Mordenti and Rescigno, (1992) Pharmaceutical Research, 9:17-25; Morenti et al., (1991) Pharmaceutical Research, 8:1351-1359; and Mordenti and Chappell, "The use of interspecies scaling in toxicokinetics" in Toxicokinetics and New Drug Development, Yacobi et al. (eds) (Pergamon Press: NY, 1989), pp. 42-96. An effective amount of the antagonist to be employed therapeutically, for example an antibody, will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the mammal. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 10 ng/kg to up to 100 mg/kg of the mammal's body weight or more per day, preferably about 1 μg/kg/day to 10

mg/kg/day. Doses may include an antibody amount any where in the range of 0.1 to 20 mg/kg of bodyweight or more preferably 1, 5, 10 mg/kg of bodyweight.

Compositions of the Invention

[0069] Antibodies produced according to the invention, can be administered for the treatment of cell prloferation, tumoregenisis, metastesis, or cancer in the form of pharmaceutical compositions.

[0070] Thus, the present invention also relates to compositions including pharmaceutical compositions comprising a therapeutically effective amount of (a) an antibody specific to angiopoietin like 4 protein (ANGPTL4) and, or (b) an antibody specific to the C terminal region of angiopoietin like 4 protein (ANGPTL4). As used herein a compound will be therapeutically effective if it is able to affect cell proliferation.

[0071] Pharmaceutical forms of the invention suitable for injectable use include sterile aqueous solutions such as sterile phosphate-buffered saline (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions and or one or more carrier. Alternatively, injectable solutions may be delivered encapsulated in liposomes to assist their transport across cell membrane. Alternatively or in addition such preparations may contain constituents of self-assembling pore structures to facilitate transport across the cellular membrane. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating/destructive action of microorganisms such as, for example, bacteria and fungi.

[0072] The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating such as, for example, lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Preventing the action of microorganisms in the compositions of the invention is achieved by adding antibacterial and/or antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

[0073] Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with several of the other ingredients

enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredient into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, to yield a powder of the active ingredient plus any additional desired ingredient from previously sterile-filtered solution thereof.

[0074] The active ingredient may be held within a matrix which controls the release of the active agent. Preferably, the matrix comprises a substance selected from the group consisting of lipid, polyvinyl alcohol, polyvinyl acetate, polycaprolactone, poly(glycolic)acid, poly(lactic)acid, polycaprolactone, polylactic acid, polyanhydrides, polylactide-co-glycolides, polyamino acids, polyethylene oxide, acrylic terminated polyethylene oxide, polyamides, polyethylenes, polyacrylonitriles, polyphosphazenes, poly(ortho esters), sucrose acetate isobutyrate (SAIB), and combinations thereof and other polymers such as those disclosed in U.S. Patent Nos. 6,667,371; 6,613,355; 6,596,296; 6,413,536; 5,968,543; 4,079,038; 4,093,709; 4,131,648; 4,138,344; 4,180,646; 4,304,767; 4,946,931, each of which is expressly incorporated by reference herein in its entirety. Preferably, the matrix sustainedly releases the antibody.

[0075] Pharmaceutically acceptable carriers and/or diluents may also include any and all solvents, dispersion media, coatings, antibacterials and/or antifungals, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, use thereof in the therapeutic compositions is contemplated.

Preferred embodiments

The angiopoietin-like 4 sustains an elevated pro-survival intracellular $O_2^{\cdot-}$:H_2O_2 ratio and confers anoikis resistance to tumor.

[0076] Tumor regression in malignant cancers remain a most significant anticancer strategy. Here we showed that elevated ANGPTL4 expression is widespread in tumors, and its suppression impairs tumor growth associated with enhanced apoptotis. ANGPTL4 interacts with integrins to modulate intracellular ROS generation that confers anoikis resistance and sustains tumor growth, underscoring ANGPTL4 as a novel player in redox cancer biology, a tumor biomarker, and thus a potential therapeutic target.

[0077] Our results demonstrate that tumor-derived ANGPTL4 confers anoikis resistance to tumors via autocrine adhesion mimicry. We show that elevated expression of ANGPTL4 is widespread in tumors. Our findings, that ANGPTL4 hijacks integrin-mediated signaling to maintain an elevated, oncogenic O_2^-:H_2O_2 ratio to confer anoikis resistance to tumor cells, identify ANGPTL4 as a novel factor in redox cancer biology and suggest anticancer strategies focused on redox-based apoptosis induction in tumors. Treatment of cancer cells with ANGPTL4-targeted RNAi or monoclonal antibodies imparts a significant decrease in *in vivo* tumor growth and induces apoptosis in 10 different cancer cell lines upon anoikis challenge. These findings should appeal to a broad audience of clinicians, cancer biologists, molecular biologists and drug designers.

Highlights

- Elevated expression of ANGPTL4 is a common feature of many human tumor types.
- ANGPTL4 binds integrin to stimulate the NADPH oxidase-dependent production of O_2^-.
- ANGPTL4 sustains a high O_2^-:H_2O_2 ratio to activate pro-survival pathways.
- Suppression of ANGPTL4 impairs tumor growth and enhances anoikis/apoptosis.

[0078] Analysis of human tumor cell lines (Figure 1a), squamous cell carcinoma biopsies (SCC) and tumor tissue arrays (Figure 3) revealed a widespread elevated ANGPTL4 mRNA and protein in epithelial tumors, regardless of anatomical site. ANGPTL4 expression increases as tumors progress from begnign to invessive/metastatic state. To assess the role of ANGPTL4 in tumorigenesis, we suppressed it by RNAi in a highly metastatic skin tumor cell, A-5RT3 (Figure 3b, Figure 4a), generating the A-5RT3$_{ANGPTL4}$ line. Injection of A-5RT3$_{ANGPTL4}$ line into immunodeficient mice yielded ~90% tumor regression, associated with increased apoptosis and reduced cell proliferation, compared to control A-5RT3$_{CTRL}$. (Figure 1c, 1d, 4b, 4c). qPCR-focused array of A-5RT3$_{ANGPTL4}$ tumor displayed increased expression of many pro-apoptotic genes, whilst cell proliferation genes were reduced (Figure 4d). Notably, immunosuppression of ANGPTL4 with a monoclonal antibody, mAb11F6C4, significantly attenuated in vivo tumor growth (Figure 1e, 4e, 4f). A-5RT3$_{ANGPTL4}$ formed 4-fold lower tumor colonies on soft agar (Figure 1f) and were more suceptable to anoikis resistance, with 40% more apoptosis within 2 h (Figure 1g).

[0079] We hypothesized that ANGPTL4 modulates oxidative stress of tumor via integrin-mediated signaling. ANGPTL4 interacted with integrins $\beta1$ and $\beta5$ but not with $\beta3$ (Figure 2a, 5a, 5b), which were blocked by either mAb11F6C4 or integrin-specific antibodies (Figure 5 c) *In situ* proximity ligation assay detected ANGPTL4-integrin complexes in vivo (Figure

2b,5d, 5e). Integrin activation triggered focal adhesion kinase (FAK) in A-5RT3$_{CTRL}$ cells and tumors, which were reduced by ~70% in A-5RT3$_{ANGPTL4}$ (Figure 2c, 2d). The expression of phosphorylated ERK1 (Figure 2e) and the number of 14-3-3/Bad complexes (Figure 2c, 2d, 5f) were reduced by ~85%, with 80% reduction of the 14-3-3β/σ proteins in the A-5RT3$_{ANGPTL4}$ induced tumors (Figure 2e). The 14-3-3 adaptor protein sequesters pro-apoptotic Bad from mitochondria to prevent apaptosis (11). Further analysis showed diminished oxidized/activated Src (8), Na+/H_ exchanger 1 (12), and increased catalase in A-5RT3$_{ANGPTL4}$ (Figure 2f, 2g), suggesting that ANGPTL4 modulates ROS generation for tumor survival.

[0080] To underscore the prevalence of ANGPTL4 in ROS generation in tumors, we examined the impact of reduced ANGPTL4 against anoikis in 12 tumor cell lines. The suppression of ANGPTL4, either by constitutive (Figure 1g), inducible (Figure 6a 6b) RNAi or immunosuppression with mAb11F6C4 (Figure 6c) resulted in 30%-60% more apoptosis within 2 hours. High intracellular ROS were reduced dose-dependantly by ANGPTL4 suppression (Figure 2h, Figure 7). Importanly, caspases 2, 3, 8, and 9 activities were increased by 3-8-fold (Figure 2i; Figure 8), indicating reduced anoikis resistance.

[0081] Altogether, ANGPTL4 is a candidate biomarker for human tumors, predominantly produced by epithelial tumor cells and a novel player in redox cancer biology. ANGPTL4 interacts with integrins to modulate ROS production, confers anoikis resistance to promote tumerogenesis, and is thus a potential therapeutic target.

[0082] It is known that in response to microenvironmental stress, such as hypoxia and inflammation, tumor cells exploit various signaling molecules to promote their growth, invasiveness and metastasis. The loss of dependence on integrin-mediated ECM contact for growth (or anoikis resistance) is an essential feature of tumor cells, yet how it is acquired is a central problem in cancer biology. Our study demonstrates a novel role for tumor-secreted ANGPTL4, which confers anoikis resistance to tumors via an autocrine adhesion mimicry that stimulates a redox-based pro-survival pathway (Figure 17). Tumor-secreted ANGPTL4 interacts with integrins in an autocrine fashion to stimulate the NADPH oxidase-dependent generation of O_2^-, promoting a high O_2^-:H_2O_2 ratio, and consequently activating downstream PI3K/PKBα and ERK activities. Our findings identify ANGPTL4 as an important novel redox player in cancer biology and suggest that anticancer therapeutics focused on redox-based apoptosis induction in tumors represent an exciting and viable strategy.

[0083] The full-length ANGPTL4 is proteolytically cleaved, giving rise to the N-terminal coiled-coil domain (nANGPTL4) and the C-terminal fibrinogen-like domain (cANGPTL4).

Depending on the tissue examined, differential expression of the various domains of ANGPTL4 was observed (Kersten et al., 2000). These observations raise the intriguing possibility that the different domains of ANGPTL4 have distinct biological functions. Furthermore, how cANGPTL4 triggers intracellular signaling to propagate its effect remains an unsolved question, hampering our understanding of the role of ANGPTL4.

[0084] We showed that only cANGPTL4 was detected and elevated in many human tumor cells, predominantly secreted by the proliferative tumor epithelial cells. cANGPTL4 specifically binds to integrins $\beta 1$ and $\beta 5$ on tumor cells and activates the FAK and Rac1, which further stimulates NADPH oxidase-mediated O_2^- production by an autocrine pathway. However, it is conceivable that in tissues/organs expressing high level of cANGPTL4 in proximity to the tumor site may trasmit a paracrine signal. Although integrins alone are not oncogenic, integrin-mediated signalings are often required to enable tumor survival and influence tumor growth (Desgrosellier and Cheresh, 2010). Our findings show that ANGPTL4-mediated integrin engagement activates ROS production, which leads to a pro-survival signal and sustained anchorage-related signals even in the absence of ECM and cell contact. The pro-oxidant intracellular environment leads to redox-mediated activation of the Src machinery, and therefore stimulates downstream PI3K/PKBα and ERK pro-survival pathways, which further triggers the 14-3-3 adaptor protein to sequester the pro-apoptotic Bad from mitochondria, which confers resistance to anoikis and favors tumor survival and growth. More importantly, our findings indicating that cANGPTL4 can modulate integrin-mediated signaling, are a pivotal step toward a better mechanistic understanding of the role of ANGPTL4.

[0085] A cell's fate is determined by the cellular redox state, through a complicated regulation mechanism, delicately maintained by intracellular ROS generators and antioxidant enzyme systems. Low or transient levels of intracellular ROS stimulate cellular signals essential for normal cellular functions. The dysregulation of intracellular ROS levels, resulting in excessive level or persistent elevation of ROS have been linked to tumor growth, invasiveness and metastasis. Indeed, elevated levels of ROS have been detected in almost all cancers (Liou and Storz, 2010). An elevated O_2^- or O_2^-:H_2O_2 ratio is particularly important for cancer cells to sustain their tumorigenicity and metastatic potential (Clement and Pervaiz, 2001; Pervaiz and Clement, 2007). Indeed, the disruption of ANGPTL4-mediated redox signaling via genetic and antibody-mediated suppression of ANGPTL4 essentially reduced the activities of FAK, Rac1 and O_2^- production. These changes resulted in an increase in tumor cells' sensitivity to anoikis and impaired tumorigenesis. ANGPTL4-stimulated

NADPH oxidase activity, leading to O_2^- production, can be inhibited by DPI and apocynin, two structurally and functionally distinct NADPH oxidase inhibitors (MacFarlane et al., 2009; Ushio-Fukai and Nakamura, 2008), but not by the mitochondrial complex I inhibitor rotenone, suggesting that O_2^- was "purposely" produced by enzymatic NADPH oxidase, rather than as a by-product of mitochondrial activity. Two survival pathways - the PKBα and ERK, which have been shown to exert anoikis-suppressing effects (Westhoff and Fulda, 2009; Zhan et al., 2004), were complementarily employed by ANGPTL4 to confer resistance to anoikis in tumor cells.

[0086] The tumor microenvironment plays a pivotal role in modulating gene expression and epithelial tumor cells' behavior. The tumor-promoting role of inflammation in the tumor microenvironment are well-recognized (Aggarwal and Gehlot, 2009). The nuclear hormone receptors PPAR, in particular PPARγ and δ/β isotypes, play major roles in the regulation of inflammation, and have been implicated in tumorigenesis (Peters and Gonzalez, 2009; Wagner and Wagner, 2010; Panigrahy et al., 2005; Murphy and Holder, 2000). Although, in our analysis of paired PNSs and SSCs, we did not observe any correlation between the expression of either PPARγ or δ/β, and their target gene ANGPTL4, we cannot exclude their involvement and/or other oncogenic pathways or cell types in the tumor microenvironment that enhanced the expression of cANGPTL4 in tumors. It is also conceivable that PPARs in cancer-associated fibroblasts play a more dominant role in the regulation of epithelial tumor growth. Indeed, we showed that PPARβ/δ-deficient fibroblasts can increase the proliferation of normal epithelial cells and SCCs via regulating interleukin-1 signaling pathway (Chong et al., 2009). The activation of interleukin-1 signaling was reported to enhance the growth of tumors, whereas its repression by the interleukin-1 receptor antagonist has an anti-tumor effect (Lewis et al., 2006). A dysregulated inflammatory response can promote tumoriogenesis and maglinancy by stimulating ROS production (Aggarwal and Gehlot, 2009). Although not examined in this study, we cannot rule out the possibility that other producers of O_2^-, such as cytosolic 5-lipooxygenase, which also requires Rac1 activation to function, may act in conjunction with ANGPTL4-stimulated NADPH oxidase activity to maintain an elevated intracellular O_2^- level for tumor growth (Chiarugi and Fiaschi, 2007).

[0087] In summary, we provided evidence that tumor cells employ ANGPTL4 to hijack integrin-mediated signaling that modulates intracellular O_2^- levels to confer anoikis resistance to tumor cells and to enhance tumorigenesis. Our findings identify ANGPTL4 as a novel tumor biomarker and redox factor in cancer biology, making ANGPTL4 a potential therapeutic target in cancer treatment.

Elevated Expression of ANGPTL4 in Various Tumor Types.

[0088] To examine the expression profile of ANGPTL4 in known human tumors, we first screened its expression pattern on two commercially available human tumor tissue arrays, which cover most of the common benign, malignant and metastatic tumors originating from various anatomic sites. By immunofluorescence with an anti-cANGPTL4 antibody, we observed a widespread elevated expression of ANGPTL4 in all epithelial tumor samples compared with the corresponding normal tissues, regardless of anatomical site of origin (Figures 10A and 18A-B). The level of immunofluorescence signal, however, varied among different types of tumor. Notably, the expression of ANGPTL4 increases as tumors progress from a benign state to an invasive/metastatic state (Figure 18C). Next, we determined the expression of ANGPTL4 on three human skin tumorigenic lines (HSC, II-4 and A-5RT3), 10 human squamous cell carcinoma biopsies (SCCs) and 13 basal cell carcinoma biopsies (BCCs) by quantitative real-time PCR (qPCR) and immunoblot analyses. Consistent with our prior results, we observed significant upregulation of ANGPTL4 mRNA and protein levels in these epithelial tumor cells when compared with the non-tumorigenic human skin line HaCaT or cognate peri-tumor normal samples (PNSs), respectively (Figures 10B-D). No difference was observed between normal skin biopsies (NS) and PNS (Figures 10C-D). Interestingly, the three SCCs expressing the highest mRNA level of ANGPTL4 corresponded with an invasive prognosis (Figure 10C), underscoring our finding in tumor tissue arrays. In addition, polyclonal antibodies against either the N- or C-termini of ANGPTL4 detected only the cANGPTL4 in these tumor lines and SSCs (Figures 10B-D and 18D-E). The expression of ANGPTL4 is upregulated by hypoxia (Belanger et al., 2002) and by peroxisome-proliferator activated receptors (PPARs) (Kersten et al., 2000). To understand the reason for the increased expression of ANGPTL4 in tumor cells, we examined the expression of hypoxia-inducible factor 1 alpha (HIF1α) and PPARs in the SCC samples. We found a concomitant upregulation of HIF1α along with ANGPTL4 in SSCs when compared with PNSs and with a Pearson correlation coefficient of 0.88 (Figures 10E and 18F). Although no clear correlation was observed between the expression of ANGPTL4 and the three PPAR isotypes (Figures 18G-I), we cannot exclude an involvement of PPARs and/or other oncogenic pathways that enhanced the expression of cANGPTL4 in tumors. These results suggested that, at least for SCC, the elevated expression of ANGPTL4 reflected the tumor's hypoxic microenvironment. Being a secreted protein highly detected in tumor cells, ANGPTL4 may perform an important paracrine or autocrine function in tumors. Therefore,

we sought to determine the source of ANGPTL4 in tumors. We isolated epithelial tumor and stromal tissues, which consist mainly of fibroblasts, from SCCs and PNSs, using laser capture microdissection (LCM). qPCR and immunoblot analyses were performed on these samples. Our results revealed that epithelial tumor cells, rather than tumor stroma, were the major contributor of ANGPTL4 in SCCs (Figure 10F), and only a low, baseline level of ANGPTL4 expression was found in normal PNS stroma and epithelia, suggesting that ANGPTL4 may have an autocrine role in tumors.

Suppression of ANGPTL4 Impairs *in Vivo* Tumor Growth.

[0089] Our findings revealed an elevated expression level of ANGPTL4 in tumors. Next, we investigated its biological relevance to tumor growth by RNA interference. Four sets of siRNAs targeting different segments of the ANGPTL4 sequence were permanently introduced into the metastatic skin tumor line A-5RT3 (Mueller et al., 2001), and the sub-line with highest knockdown efficiency (designated A-5RT3$_{ANGPTL4}$) was selected for subsequent studies. A non-targeting scrambled siRNA was also integrated into A-5RT3 (designated A-5RT3$_{CTRL}$), serving as a negative control. ANGPTL4 mRNA and protein levels were successfully suppressed by > 85% in A-5RT3$_{ANGPTL4}$ when compared with the parental control A-5RT3 or the scrambled control A-5RT3$_{CTRL}$ (Figure 11A). The induction of interferon responses has been reported as a challenge to the specificity of some RNA interference approaches (Bridge et al., 2003). To test whether the RNAi-mediated silencing of ANGPTL4 was associated with interferon responses, we measured the expression of some key interferon response genes by qPCR. No induction of Oas1, Oas2, Mx1 or Isgf3γ was detected in the A-5RT3$_{ANGPTL4}$ when compared with either wild-type, untreated A-5RT3or A-5RT3$_{CTRL}$ (Figure 19A), verifying that our RNAi experiment did not produce an off-target effect. As expected, the injection of A-5RT3$_{CTRL}$ cells into immunodeficient mice induced large primary tumors (~1000 mm^3) in all five mice at week eight, however A-5RT3$_{ANGPTL4}$-induced tumors showed a 90% reduction in tumor growth (Figures 11B-C). A-5RT3$_{ANGPTL4}$-induced tumor growth was similarly reduced, albeit a 40% reduction, when mice were implanted with increasing number of tumor cells (Figure 19B). To strengthen the above observation, we implanted B16F10 cells subcutaneously into ANGPTL4-knockout (KO) and control (WT) mice. WT and KO mice were maintained in a C57BL/6J background and B16F10 melanoma was derived from the same background. Notably, B16F10 tumor cells implanted in KO mice grew significantly slower than those implanted in WT mice; at 15 days post implantation, the average tumor volume in KO mice was ~6-fold smaller than that in WT mice (Figure 11D). The injection of ANGPTL4-knockdown (B16F10$_{ANGPTL4}$) cells

into KO mice induced little tumor growth, and showed similar growth profile in WT mice to control B16F10 ($B16F10_{CTRL}$)-induced tumors in KO mice (Figure 11D). Conversely, WT mice implanted with $B16F10_{CTRL}$ and intravenously injected thrice weekly with recombinant N-terminal histidine-tagged recombinant cANGPTL4 showed greater tumor growth, the average tumor volume in cANGPTL4-treated mice was ~3-fold larger than PBS-treated mice (Figures 11E and 19C-D). $B16F10_{ANGPTL4}$-induced tumor growth was diminished in PBS-treated mice when compared to cANGPTL4-treated mice (Figure 11E). Next, we reasoned that treating mice injected with A-$5RT3_{CTRL}$ cells with an antibody that interferes with the action of ANGPTL4 will recapitulate the observation made with A-$5RT3_{ANGPTL4}$. To this end, the monoclonal human cANGPTL4-directed antibody mAb11F6C4 was identified and produced for our immunotherapy experiment based on its superior k_{on}, k_{off} and K_D values, as determined by surface plasmon resonance (SPR) (Figure 11F). Notably, immunosuppression of ANGPTL4 with mAb11F6C4 significantly attenuated *in vivo* tumor growth in immunodeficient mice, compared with control IgG-treated mice (n = 6 each group) (Figures 11G-H). Immunoblot and immunofluorescence analysis of A-$5RT3_{ANGPTL4}$-induced tumor biopsies indicated significantly reduced cell proliferation and enhanced cell apoptosis when compared with A-$5RT3_{CTRL}$-induced tumors (Figures 11I-J). A qPCR-focused array of A-$5RT3_{ANGPTL4}$-induced tumor biopsies further suggested increased expression of many pro-apoptotic genes, whereas expression of cell proliferation genes were diminished (Figure 11K; Table 1). Altogether, these observations clearly supported a tumor-promoting role of cANGPTL4.

ANGPTL4-Deficient Tumor Cells Showed Increase Susceptibility to Anoikis.

[0090] Anchorage-independent growth or anoikis resistance of tumor cells, a hallmark of tumor malignancy (Hanahan and Weinberg, 2000), can be investigated by tumor colony formation in soft agar and anoikis assays, which are well-established *in vitro* approaches to study and predict self-renewal and metastatic potentials of *in vivo* tumor cells (Salmon, 1984). Underscoring our *in vivo* findings, the colony-forming potential of A-$5RT3_{ANGPTL4}$ was dramatically undermined and formed significantly (~85%) fewer tumor colonies on soft agar when compared with A-$5RT3_{CTRL}$ (Figure 12A). Furthermore, A-$5RT3_{ANGPTL4}$ was also more susceptible to anoikis, having 30% more apoptotic A-$5RT3_{ANGPTL4}$ cells, as well as significantly enhanced activities from caspases 2, 3, 8 and 9 when compared with A-$5RT3_{CTRL}$ after 2 h of anoikis (Figure 12B-C). The addition of exogenous recombinant cANGPTL4 reduced the apoptotic index of A-$5RT3_{ANGPTL4}$ in a dose-dependent manner (Figure 12D). Similarly, ANGPTL4 deficiency in human keratinocytes rendered these cells

~50% more susceptible to anoikis when compared to control keratinocytes, suggesting that low amount of ANGPTL4 was also necessary to confer anoikis resistance in normal epithelial cells (Figure 20A). No difference in apoptotic index was observed with adhered A-5RT3 and keratinocytes (Figure 20A-B).

ANGPTL4 Interacts with Integrins β1 and β5.

[0091] Our above findings indicated that ANGPTL4 endows tumor cells with resistance to anoikis and therefore sustain their growth, but how ANGPTL4 mediates this process remains a central question in our understanding of ANGPTL4 in cancer biology. Previous studies have revealed that anoikis is an integrin-dependent process (Chiarugi, 2008; Eble and Haier, 2006; Zhan et al., 2004), thus we hypothesize that ANGPTL4 also exerts its role in tumor cells through integrins-mediated signaling. First, we examined if cANGPTL4 can interact with integrin. Indeed, results obtained from SPR and ELISA assays showed that ANGPTL4 specifically interacts with integrins β1 and β5, but not with β3 (Figure 12E-F), which were blocked by either mAb11F6C4 or integrin-specific antibodies (Figures 12G-H and 20D-G). ANGPTL4 deficiency did not affect the expression of integrins β1, β3 and β5 (Figure 20H). An *in situ* proximity ligation assay (PLA) detected ANGPTL4-integrin complexes both in A-5RT3$_{CTRL}$ cells and A-5RT3$_{CTRL}$-induced tumor biopsies (Figures 20I and 12I), confirming that this interaction also exists *in vivo*. Further investigation revealed that integrin activation by ANGPTL4 binding triggered focal adhesion kinase (FAK) in A-5RT3$_{CTRL}$ cells and tumors, which were reduced by > 70% in A-5RT3$_{ANGPTL4}$ (Figures 12J and 20J). All of these findings were further corroborated by results from immunodetection on tumor biopsies (Figure 12K). Our findings suggest that ANGPTL4 secreted by epithelial tumor cells acts in an autocrine manner to hijack the integrin/FAK-regulated pathway to confer anoikis resistance to tumors, and thus sustain tumor growth.

ANGPTL4 Elevates O_2^- Level and Maintains a High O_2^-:H_2O_2 Ratio in Tumor Cells.

[0092] Reactive oxygen species (ROS; e.g. O_2^- and H_2O_2) have long been recognized as important second messengers, functioning in the relay of intracellular signals in normal and cancer cells (Liou and Storz, 2010; Thannickal and Fanburg, 2000). ROS can be regulated through integrin engagement and an elevated O_2^- level or relatively high O_2^-:H_2O_2 ratio allows tumor cells to survive and to avoid anoikis (Pani et al., 2009; Pervaiz and Clement, 2007; Pervaiz et al., 1999). In this regard, we asked whether ANGPTL4-integrin interaction can regulate ROS production in tumor cells. Using electron paramagnetic resonance spectroscopy (EPR) in combination with 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO) spin trapping, we measured a significant decrease in O_2^- level in A-

5RT3$_{ANGPTL4}$ when compared with A-5RT3$_{CTRL}$ (Figure 13A-B), suggesting ANGPTL4 is vital in sustaining O_2^- production in tumor cells. To determine the source of O_2^-, similar experiments were performed using specific inhibitors that block the mitochondrial respiratory chain complex I and membrane-bound NADPH oxidase, which are two major producers of O_2^- in mammalian cells (Giannoni et al., 2008). Treatment of tumor cells with rotenone, a mitochondrial respiratory chain complex I inhibitor (Irani et al., 1997), did not alter their cellular O_2^- level (Figure 13A-B) suggesting that such a complex has little role in generating O_2^- in tumors. Further excluding mitochondria as the source of ANGPTL4-mediated O_2^- generation, our qPCR analysis showed no change in the expression of selected genes in the methionine/homocysteine metabolic cycle (Figure 21A), as previously studied in db/db diabetic rodent hepatocytes (Wang et al., 2007). In contrast, O_2^- level was significantly abrogated by using two different NADPH oxidase inhibitors (Ushio-Fukai and Nakamura, 2008), namely, diphenylene iodonium (DPI) and apocynin (Figure 13A-B). Reactive oxygen species generated through the involvement of the small GTPase Rac1 and NADPH oxidase upon integrin engagement exert a mandatory role in transmitting a pro-survival signal that ensures the tumor cells escape from anoikis (Giannoni et al., 2008; Joneson and Bar-Sagi, 1998). In accordance with these results, comparative immunoblot analysis of anti-cANGPTL4 immunoprecipitates from A-5RT3$_{CTRL}$- and A-5RT3$_{ANGPTL4}$-induced tumor lysates detected integrins β1 and β5, along with phosphorylated FAK and active GTP-bound Rac1, in A-5RT3$_{CTRL}$-induced tumor, but were significantly reduced in A-5RT3$_{ANGPTL4}$-induced tumor (Figure 12K). To further validate the relevance of Rac1 in ANGPTL4-mediated O_2^- production, we next transiently transfected A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ with dominant-negative Rac1 (T17N) and constitutively active Rac1 (G12V), respectively. We measured a significantly diminished O_2^- level in the former system and, conversely, an obvious rescued level of O_2^- production in the latter one. The percentage of inhibition and recovery was consistent with the ~65% transfection efficiencies, as estimated using a GFP-expressing vector. The requirement of Rac1 suggested a Nox-dependent mechanism. Thus, we examined the expression of Nox1 and Nox 2 in A-5RT3 (Figure 21B). Nox 3 is expressed predominantly in the inner ear and is involved in the biogenesis of otoconia/otolith (Paffenholz et al., 2004). Next, we performed Nox1 and Nox2 knockdown (Nox1 kd and Nox2 kd, respectively) in A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ (Figure 21C), and measured O_2^- level using EPR (Figure 13A-B). The results indicated that Nox 1 NADPH oxidase is the predominant producer of ANGPTL4-mediated O_2^- generation in tumor cells. As expected, O_2^- was completely abolished when treated with the superoxide scavenger Tiron, which

serves as a negative control for superoxide measurement (Figure 13A-B). These data were reproduced by a reliable chemiluminescence method using 2-methyl-6-(4-methoxyphenyl)-3, 7-dihydroimidazo[1, 2-a]pyrazin-3-one hydrochloride (MCLA; Figure 13C) (Münzel et al., 2002). Next, we measured the level of H_2O_2 in tumor cells in the presence of a specific catalase inhibitor, 3-amino-1, 2, 4-triazole (Chance et al., 1979; Wagner et al., 2005). H_2O_2 levels in A-5RT3$_{ANGPTL4}$ were significantly higher when compared with A-5RT3$_{CTRL}$ (Figure 13D). Nox1 knockdown did not affect the H_2O_2 level, suggesting that ANGPTL4 modulated H_2O_2 production via a linked as-yet-unknown mechanism (Figure 21D). Notably, the lower O_2^- level and O_2^-:H_2O_2 ratio was concurrent with threefold more apoptosis and significantly enhanced caspase activities within 2 h of anoikis in A-5RT3$_{ANGPTL4}$ when compared with A-5RT3$_{CTRL}$ (Figures 13A-D and 12B-C). In concordance, we observed a reduced O_2^- level in A-5RT3$_{ANGPTL4}$-induced tumors when compared with A-5RT3$_{CTRL}$-induced tumors as determined by EPR (Figure 13E-F) which was associated with increased apoptosis (Figure 11I-K).

[0093] To underscore the relevance of these findings to other cancers, similar experiments were performed using the breast cancer line MDA-MB-231, after using the mononclonal antibody mAb11F6C4 to dose-dependently neutralize endogenous cANGPTL4. We showed earlier that mAb11F6C4 was able to block cANGPTL4-integrin interaction (Figures 12G-H and 20D-G). Consistent with the above results, the immunosuppression of cANGPTL4 in MDA-MB-231 reduced the O_2^- level (Figure 13G-I), lowered the O_2^-:H_2O_2 ratio (Figure 13J), and enhanced apoptosis and caspase activities (Figure 13K-L). The knockdown of Nox1 (Figure 21E), but not Nox2, reduced ANGPTL4-mediated O_2^- production (Figure 13G-I) with little effect on H_2O_2 production (Figure 21F). Taken together, these findings indicated that ANGPTL4 could protect tumor cells from anoikis via an NADPH oxidase-dependent O_2^- generation mechanism.

ANGPTL4-mediated O_2^- Activates Src, PI3K/PKBα and ERK Survival Pathways

[0094] Reports have shown that ROS produced via integrin engagement oxidizes and activates Src, which stimulates the ERK and PKBα pro-survival pathways (Giannoni et al., 2008; Giannoni et al., 2009; Pani et al., 2009; Werner and Werb, 2002). Both pathways regulate the subcellular localization or stability of BH3-only apoptotic proteins (e.g. Bad and Bim), essential for executing anoikis (Bouillet and Strasser, 2002). Thus, we asked whether ANGPTL4-integrin engaged O_2^- generation employs these downstream signaling pathways to modulate tumor cell behavior. Indeed, immunoblot analysis revealed significantly

diminished expression of oxidized/activated Src, phosphorylated PKBα and ERK1 in A-5RT3$_{ANGPTL4}$-induced tumors and A-5RT3$_{ANGPTL4}$ cells (Figures 14A and left panel of 14B). Similar immunoblot analysis performed in the presence of DPI and with Nox1 knockdown cells, which attenuate ANGPTL4-mediated O_2^- production, found severely diminished Src, PKBα and ERK1 activations, emphasizing the role of O_2^- in their activities (Figure 14B). The inhibition of PI3K by LY29402 and Wortmannin, a pivotal upstream mediator of PKBα, caused significantly (four-fold) more apoptosis of tumor cells within 2 h of anoikis challenge, reaching levels comparable to those of A-5RT3$_{ANGPTL4}$ (Figure 14C). In addition, inhibition of MEK1/2, the upstream signal of ERK1, by PD98059 also resulted in a significant enhancement of apoptotic cell numbers upon anoikis challenge, albeit to a lesser extent (~50%) when compared with PI3K inhibitors (Figure 14C). These results suggested that PI3K/PKBα and ERK1/2 downstream survival pathways were modulated and exploited by ANGPTL4 engagement in tumor cells, the former being the predominant path.

[0095] The 14-3-3 adaptor protein is known to act downstream of the survival pathways by sequestering pro-apoptotic Bad from the mitochondria to prevent apoptosis (She et al., 2005). In agreement with these previous findings, the number of 14-3-3/Bad complexes and 14-3-3β/σ proteins were significantly reduced by ~70% in A-5RT3$_{ANGPTL4}$-induced tumors (Figure 14D-F). The Na^+/H^+ exchanger 1 (NHE), which positively influences cell proliferation by maintaining an alkaline intracellular environment (Akram et al., 2006; Shibanuma et al., 1988), was diminished in A-5RT3$_{ANGPTL4}$-induced tumors (Figure 14D), indicating that NHE plays a subsidiary role to ANGPTL4-mediated tumor cell growth. Upon oxidant challenge in tumor cells, the induction of superoxide dismutase (SOD) expression was muted, allowing tumor cell proliferation (Oberley, 2001; Pervaiz and Clement, 2007). In agreement with these studies, we found that expression of the cytosolic Zn/CuSOD was significantly enhanced in A-5RT3$_{ANGPTL4}$-induced tumors (Figure 14D), which indirectly contribute to the reduced O_2^-:H_2O_2 ratio in ANGPTL4-deficient tumor cells (see Figures 13D, J and 21D, F).

ANGPTL4 Deficiency Abrogates O_2^- Production and Sensitizes Cancer Cells to Anoikis

[0096] Our results revealed that the suppression of ANGPTL4, either by constitutive RNAi (Figure 13A-C) or immunosuppression with mAb11F6C4 (Figure 13G-I) resulted in a dose-dependent reduction of O_2^- levels. To underscore the importance of ANGPTL4 in the regulation of O_2^- production, maintenance of high O_2^-:H_2O_2 ratio, and hence tumor survival, we examined the impact of reduced ANGPTL4 against anoikis in nine different cancer cell

lines, in addition to A-5RT3 and MDA-MB-231. Treatment with mAb11F6C4, which blocked cANGPTL4-integrin interaction, resulted in a dose-dependent reduction of O_2^- levels (40-80% for 6 μg/mL mAb11F6C4; Figure 15A), a reduction in O_2^-:H_2O_2 ratio (70-90% for 6 μg/mL mAb11F6C4; Figure 15B), a 3-8 fold increase in the activities of caspases 2, 3, 8 and 9 (Figure 16A) and 30-60% more apoptotic tumor cells (Figure 16B), all indicating weakened anoikis resistance and further corroborating our previous observations on A-5RT3 and MDA-MB-231. Higher percentage of apoptotic tumor cells was also observed using inducible RNAi against ANGPTL4 in MDA-MB-231 (Figure 16C). These findings indicated that ANGPTL4-mediated O_2^- production for anoikis resistance may be a common feature in tumor cells.

Table 1. Relative fold change of gene expressions in A-5RT3$_{ANGPTL4}$-induced tumors as compared with that of A-5RT3$_{CTRL}$-induced tumors, related to Figure 2 and 11

Down-regulated (> 2-fold)		
Gene	**A-5RT3$_{CTRL}$**	**A-5RT3$_{ANGPTL4}$**
Diablo	1.000	0.070
Ccnd1	1.000	0.102
Ccna2	1.000	0.119
Xiap	1.000	0.120
Pcna	1.000	0.177
Cox10	1.000	0.223
Birc2	1.000	0.247
Ki67	1.000	0.269
Birc3	1.000	0.345
Cdk5	1.000	0.498
Mcl1	1.000	0.500
Cdk4	1.000	0.549

Up-regulated (> 2-fold)		
Gene	**A-5RT3$_{CTRL}$**	**A-5RT3$_{ANGPTL4}$**
Casp7	1.000	1.927
Bid	1.000	2.051
Bbc3	1.000	2.075
Perp	1.000	2.246
Parp1	1.000	2.308
Pxn	1.000	2.947
Bcl2l1	1.000	3.112
Cdkn1c	1.000	3.609
Fas	1.000	6.171
Chuk	1.000	6.353
Bax	1.000	8.363
Casp1	1.000	10.499
Casp2	1.000	10.560
Cdkn1a	1.000	13.037
Bcl2l2	1.000	14.671
Casp10	1.000	24.740

Note: The Gene expression levels in A-5RT3$_{CTF}$

Table 2 Sequences of ANGPTL4, Nox1, Nox2 and Control siRNAs

siRNA	Sense Primer (5' → 3')	Antisense Primer (5' → 3')
ANGPTL4 set 1*	AAAGCTGCAAGATGACCTCAGATGGAGGCTG	AAAACAGCCTCCATCTGAGGTCATCTTGCAG

ANGPTL4 set 2#	TCGAG**GCAGCACCTGCGAATTCAGCATCTGCA TTCAAGAGATGCAGATGCTGAATTCGCAGGTG CTGCTTTTTT**ACGCGTA	AGCTTACGCGT**AAAAAGCAGCACCTGCGAATT CAGCATCTGCATCTCTTGAATGCAGATGCTGA ATTCGCAGGTGCTG**CC
Nox1	AAAGGGCCACAGATGGCTCCCTTGCCTCCAT	AAAAATGGAGGCAAGGGAGCCATCTGTGGCC
Nox2	AAAGGGCCAGATGTTCTTTCTACAGAAGAAT	AAAAATTCTTCTGTAGAAAGAACATCTGGCC
Mouse ANGPTL4	AAAGCTGTGAGATGACTTCAGATGGAGGCTG	AAAACAGCCTCCATCTGAAGTCATCTCACAG
Control siRNA	AAAGCTGTCTTCAAGCTTGATATCGAAGACTA	AAAATAGTCTTCGATATCAAGCTTGAAGACAG

*ANGPTL4 Set 1 siRNA used for lentivirus-mediated RNA interference (SEQ ID NO. 4 & 5).

ANGPTL4 set 2 shRNA was cloned into pSingle-tTS-shRNA vector (Clontech) and used for doxycycline-inducible knockdown in MDA-MB-231 cells (SEQ ID NO. 6 & 7).

Table 3 Sequences of Quantitative Real-time PCR (qPCR) Primers

GenBank Accession	Official Symbol	Sense Primers (5' → 3')	Antisense Primers (5' → 3')
NM_004324	Bax	GGGTGGTTGGGTGAGACTC	AGACACGTAAGGAAAACGCATTA
NM_014417	Bbc3	GACCTCAACGCACAGTACGAG	AGGAGTCCCATGATGAGATTGT
NM_138578	Bcl2l1	TGCGTGGAAAGCGTAGACAAG	GCTGCTGCATTGTTCCCATA
NM_004050	Bcl2l2	GCGGAGTTCACAGCTCTATAC	AAAAGGCCCCTACAGTTACCA
NM_001196	Bid	GACAGCATGGACCGTAGCATC	AGGTGCGTAGGTTCTGGTTAATA
NM_001166	Birc2	GTTTCAGGTCTGTCACTGGAAG	TGGCATACTACCAGATGACCA
NM_182962	Birc3	TCCTGGATAGTCTACTAACTGCC	GCTTCTTGCAGAGAGTTTCTGAA
NM_033292	Casp1	TCCAATAATGGACAAGTCAAGCC	GCTGTACCCCAGATTTTGTAGCA
NM_001230	Casp10	ATTGGTCCCAAGACATGAAGAC	TGTTCCCTGTTTGTCCACTCT
NM_032982	Casp2	AAACGAGGTTCCTGGTACATCG	TCCTTGATAAGTGCGTTCACC
NM_033340	Casp7	AGTGACAGGTATGGGCGTTC	GAGGTTGCAGTCTTCCGAGAT
NM_001237	Ccna2	GATGGTAGTTTTGAGTCACCACA	CACGAGGATAGCTCTCATACTGT
NM_053056	Ccnd1	GCTGGAGCCCGTGAAAAAGA	CTCCGCCTCTGGCATTTTG
NM_000075	Cdk4	CAGATGGCACTTACACCCGTG	GCAGCCCAATCAGGTCAAAGA
NM_004935	Cdk5	GCCGCAATGTGCTACACAG	GAGTAACAGCGGACGGGAATC
NM_000389	Cdkn1a	GTCACTGTCTTGTACCCTTGTG	CGGCGTTTGGAGTGGTAGAAA
NM_000076	Cdkn1c	ACATCCACGATGGAGCGTC	GGAAGTCGTAATCCCAGCGG
NM_001278	Chuk	CAGCCATTTACCTGGCATGAG	GAGGGTCCCAATTCAACATCAA
NM_001303	Cox10	CCAGCAAGTAAGACCCAAGCC	TCATCTCTTTCCACCGCTTTTC
NM_019887	Diablo	GGTACAGACAGTGTTTGTGTGT	CTACTAAGGGAATGAGGCTCTGA
NM_000043	Fas	TATCACCACTATTGCTGGAGTCA	ACGAAGCAGTTGAACTTTCTGTT
NM_002417	Ki67	TGTTCCCACTACACAATGTCTTG	ACTTACGCGAGACCAACAGTT
NM_021960	Mcl1	GTGCCTTTGTGGCTAAACACT	AGTCCCGTTTTGTCCTTACGA
NM_001618	Parp1	GATGCCTATTACTGCACTGGG	CGGTCCTGCTTTTTAACCTTCAA
NM_022121	Perp	CAACCCTGCTGTCACTTACAT	AGGTCATCTTCGTAGTTGGGG
NM_182649	Pcna	ACACTAAGGGCCGAAGATAACG	CGGCATATACGTGCAAATTCAC
NM_002859	Pxn	GCGGACTTGGAGTCTACCAC	TCCAGTTGGGTATGAGTAGGG
NM_001167	Xiap	GACAGGCCATCTGAGACACAT	GGGGTTAGGTGAGCATAGTCTG
NM_000687	Ahcy	GCATCCGAGGCATCTCTGAG	GCCATAGAGGTTGTCAAACTTGC
NM_001713	Bhmt	GACACCTTCATACCTTAGCTGC	ACAGGTTTACCGGATGCTATCAA
NM_012260	Hacl1	CCTTCTTATCATCGGGAAAGGTG	CCCATAGGGGTGGGCAAAAAT
NM_000221	Khk	GCTATTCTGTGGACCTACGCT	AGTATAGGATGGTGCGGCTAC
NM_000429	Mat1a	CATCAAGCACATCGGCTACGA	CCGAACATCAAACCCTGATCTC
NM_000274	Oat	TGCTGTCAACCAAGGGCATT	GCCTCCACTCCTGTATTCATAGG
NM_000988	L27	TGATGGCACCTCAGATCGC	AGAGTACCTTGTGGGCATTAGG

Note: Melt curve analysis was included to assure that only one PCR product was formed.

Experimental Reagents.

[0097] Antibodies were used: cyclinD1, keratin 10, integrins ß1 and ß5, involucrin (Chemicon); caspase-3 (R&D Systems); PCNA, transglutaminase 1 (TGase 1), ß-tubulin, 14-3-3 ß, 14-3-3σ, catalase, ERK1/2, p(T202/Y204)ERK1/2 (Santa Cruz Biotechnology); c-Src, p(Y416)Src, FAK, p(Y925)FAK (Cell Signaling Technology); pan-14-3-3 and BAD (Abcam); Bax and Cleaved PARP (Millipore); Ki67 (NovaCastra); secondary Alexa488-conjugated antibodies (Invitrogen); secondary HRP-conjugated antibodies (Santa Cruz Biotechnology). Rat tail collagen type I (BD Biosciences, USA), pFIV lentivirus-based siRNA vector and packaging kit (System acetyl ester was from Molecular Probes.

Transfection reagent ExGen 500 and restriction enzymes were from Fermentas. Monoclonal and polyclonal antibodies against the C-terminal region human (186-406 amino acids) and mouse (190-410 amino acids) ANGPTL4 were produced according to standard procedures. Unless specified, all reagents were obtained from Sigma.

Cell Culture.

[0098] HaCat is a non-tumor human keratinocyte cell line, II-4 and A-5RT3 are tumoriogenic HaCat derivatives were provided by the German Cancer Research Center. HSC is a human squamous cell carcinoma cell line was provided by Prof. Aso (Yamagata University School of Medicine, Japan). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone). All cells were cultured at 37°C, 5% CO_2 and 75% humidified incubator.

Human Tumor Samples.

[0099] Human squamous cell carcinoma biopsies along with their paired peri-tumor normal samples were provided by the National Skin Centre of Singapore. Whole SCCs and PNSs samples, inclusive of epithelia and stroma, were subjected to total protein or RNA extraction for immunoblotting or qPCR analyses. For LCM samples, epithelial and stromal fractions were microdissectioned from 8-μm-thick sectioned tissues using PALM Microbeam Axio Observer Z1 (Carl Zeiss) and qPCR was performed as described[1].

[00100] Commercial tumor tissue arrays #MTU951 and #MET961 (Pantomics, Inc., USA) were utilized to study the expression profile of ANGPTL4 in a large known human tumor set by immunofluorescence imaging. The #MTU951 human tumor tissue array contains 40 tumor types, covering most of the common benign, malignant and metastatic tumors originated from 27 anatomic sites, and the #MET961 human cancer metastasis tissue array consists of 48 cases of metastatic cancers from > 8 anatomic sites. The two tissue arrays were probed with anti- cANGPTL4 polyclonal antibody followed by Alexa488 goat-anti-rabbit IgG. Images were taken by an inverted microscope (ECLISPSE TE2000-U; Nikon) with equal exposure and gain. The 3D heatmaps were generated using IMARIS software (Bitplane Scientific Software). In the heatmaps, the X-Y axis represents the length and width, while the Z axis represents the IF intensity.

[00101] Human basal cell carcinoma biopsies (BCCs) and squamous cell carcinoma biopsies (SCCs) along with their paired peri-tumor normal samples (PNSs) were provided by

Dr. Pan, Dr. Tan (National Skin Centre, Singapore) and purchased from Asterand plc, USA. BCC, SCC and PNS samples, inclusive of epithelia and stroma, were subjected to protein and RNA extraction for immunoblotting and qPCR analyses, respectively.

[00102] Commercial tumor tissue arrays #MTU951 and #MET961 (Pantomics, Inc., USA) were utilized to study the expression profile of ANGPTL4 in a large human tumor set by immunofluorescence (IF) imaging. The #MTU951 human tumor tissue array contains 40 tumor types, covering most of the common benign, malignant and metastatic tumors originating from 27 anatomic sites, and the #MET961 human cancer metastasis tissue array consists of 48 cases of metastatic cancers from >8 anatomic sites. The two tissue arrays were probed with anti-cANGPTL4 polyclonal antibody followed by Alexa488 goat-anti-rabbit IgG. Images were taken using MIRAX MIDI with Plan-Apochromatic 20x/0.8 objective, with equal exposure and gain and each images automatically stitched by MIRAX Scan software (Carl Zeiss). The 3D heatmaps were generated using IMARIS software (Bitplane Scientific Software). In the heatmaps, the X-Y axes represent the length and width, whereas the Z axis represents the IF intensity. The gray value (IF intensity) was obtained from three biopsies using TissueQuest software (TissueGnostic GmbH).

Suppression of ANGPTL4 by RNA Interference (RNAi).

[00103] Four sets of siRNAs against human ANGPTL4 and a scrambled sequence as control (Table 2) were subcloned into the pFIV-H1/U6-puro pFIV/siRNA lentivirus system. The correct pFIV siRNA constructs were verified by sequencing using H1 primer. Pseudovirus purification and transduction were performed[1]. ANGPTL4-knockdown tumor cells were enriched by puromycin selection for 1 week. The A-5RT3 sub-cell line designated A-5RT3 $_{ANGPTL4}$, with the highest knockdown efficiency was chosen in this study, and the non-targeted siRNA transduced line was denoted as A-5RT3$_{CTRL}$. The expression of endogenous ANGPTL4 in MDA-MB-231 cells was also suppressed using tetracycline-inducible pSingle-tTS-shRNA vector (Clontech). Knockdown efficiency of ANGPTL4 and relative expression level of indicated genes were determined by qPCR and immunoblotting.

Total RNA Isolation and Quantitative Real-time PCR (qPCR).

[00104] Total RNA was extracted and qPCR was performed1. Expression was related to the housekeeping gene 60S ribosomal protein L27 (RPL27) which did not change under any of the experimental conditions studied. The sequence of primers is available in Table 3.

For focused mRNA array, genes whose expression was changed significantly (>2-fold) were listed and heatmaps were generated using Orange Canvas 1.0 software.

Immunoblotting.

[00105] Total protein was extracted from cells, or tumor tissues with ice-cold lysis buffer (20 mM $Na_2H_2PO_4$, 250 mM NaCl, 1% Triton-100, 0.1% SDS). Equal amount of protein extracts were resolved by SDS-PAGE and electrotransferred onto PVDF membranes. Membranes were processed according to standard procedure and proteins were detected by chemiluminesence (Millipore, USA). ß-tubulin was used as loading and transfer control.

***In vivo* tumorigenecity assay.**

[00106] Five BALB/c athymic nude female mice (20-22g), aged 5-6 weeks, were purchased from A*STARBiological Resources Centre (Singapore), and maintained in panthogen-free conditions. The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC0092), Nanyang Technological University, and all experiments were carried out in strict compliance with their regulations. A total of 5×10^5 cells ($A\text{-}5RT3_{CTRL}$ or $A\text{-}5RT3_{ANGPTL4}$) was injected subcutaneously into the interscapular region of each nude mouse. Injection site was rotated to avoid site bias. The injected tumor cells were allowed to grow for 8 weeks, The subcutaneous xenograft tumors were measured externally with a vernier caliper every other day, and tumor volume was estimated by using the equation, $V = (L \times W^2)/2$, where L is the length of the major axis of the tumor, and W is the length of the minor axis. Mice were sacrificed at the end of the experiment, and their tumors were harvested for further analysis.

[00107] For the antibody treatment, 6 nude mice were implanted with $A\text{-}5RT3_{CTRL}$ as above. One week post implantation, 30mg/kg/week of either mAb11F6C4 or isotype control IgG were intravenously administrated once weekly for 4 weeks. The dose of antibody and delivery mode was consistent with studies using mAb14D12, another anti-ANGPTL4 mAb[2]. Mice were sacrificed after treatments and tumors were harvested for further analyses. Laser scanning microscope with a Plan-Apochromat 63x/1.40 Oil objective and ZEN 2008 software (Carl Zeiss).

Soft Agar and Anoikis Assay.

[00108] $A\text{-}5RT3_{CTRL}$ and $A\text{-}5RT3_{ANGPTL4}$ cells were used in soft agar assay. 0.6% Noble agar (Sigma Aldrich) in DMEM with 10% FBS was allowed to solidify in 6-well plate, and 1×10^4 cells were plated in 0.3% Noble agar in DMEM with 10% FBS on top.

Tumor cell colonies were stained with 1 mg/ml thiazolyl blue tetrazolium in PBS after 4 weeks.

[00109] Cells were subjected to anoikis assay[3]. Briefly, anoikis was induced by forced suspension where 5.0 x 10^5 cells were seeded onto 1.0% serum-free DMEM equilibrated agarose in the presence either 10μg/ml of pre-immune IgG or mAbIIF6C4. For MBA-MD-231, the cells were exposed to 1 μg/ml doxycyline for 24 h to knockdown ANGPTL4 prior anoikis. Cells were harvested at indicated time points, and analyzed for apoptosis by FACS analysis.

[00110] A-5RT3$_{CTRL}$ and A-5RT3$_{ANGPTL4}$ cells were used in soft agar assay. 0.6% Noble agar (Sigma Aldrich) in DMEM with 10% FBS was allowed to solidify in 6-well plate, and 1 × 10^4 cells were plated in 0.3% Noble agar in DMEM with 10% FBS on top. Tumor-cell colonies were stained with 1 mg/ml thiazolyl blue tetrazolium in PBS after four weeks.

[00111] Cells were subjected to an anoikis assay. Briefly, anoikis was induced by forced suspension, wherein 5.0 × 10^5 cells were seeded onto 1.0% serum-free DMEM equilibrated agarose in the presence of either 10 μg/ml of pre-immune IgG or mAb11F6C4. For MBA-MD-231, the cells were exposed to 1 μg/ml doxycyline for 24 h to knockdown ANGPTL4 prior anoikis. For rescue experiments, cells were subjected to anoikis in the presence of either indicated concentrations of exogenous recombinant cANGPTL4 or vehicle (PBS). Cells were harvested at indicated time points, and analyzed for apoptosis by FACS analysis. Apoptotic index of attached cells were determined immediately after harvesting with trypsin.

Membrane protein extraction.

[00112] HEK293T cells were transferred with either empty mammalian expression vector pEF1-mycA (Invitrogen) or vector carrying cDNAs encoding human integrins ß1, ß3 and ß5 by means of ExGen 500. Forty-eight hours post-transfection, cell membranes were first isolated using ProteoExtractNative Protein Extraction Kit (Calbiochem) and subjected to enrichment by sucrose step gradient[4]. The proteins were displayed against PBS prior to SPR analysis.

Fluorescence-activated cell sorting (FACS).

[00113] Cells were analyzed for apoptosis using the Annexin V-FITC apoptosis kit (BD Pharmingen) according to manufacturer's instructions. After 2 h of anoikis challenge,

cells were harvested, washed in cold PBS and strained with annexin V-FITC and propidium iodide for 15 min at room temperature in the dark. Cells were resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCL_2$), and subjected to flow cytometry on a FACS Calibur (Becton Dickinson). Data were analyzed using the CellQuest software (Becton Dickinson). The analyser threshold was adjusted on the flow cytometer channel to exclude most of the subcellular debris to reduce the background noise. The totality of Annexin V^+/PI^- (early apoptosis) and Annexin V^+/PI^+ cells (late apoptosis) were considered apoptotic.

Surface Plasmon Resonance (SPR analysis.

[00114] Purifided fibrinogen-like fragment of ANGPTL4 (cANGPTL4) was immobilized onto ProteOn GLC chip by amine coupling as recommended by the manufacturer (Bio-Rad). Different concentrations of integrins were introduced into the GLC chip at a flow rate of 25 μl/min for 5 min with running buffer (50mM Tris, pH8.0, 100 mMNaCl). Polyclonal anti-cANGPTL4 antibodies against the immobilized cANGPTL4 determined the Rmax value to be 423.1 resonance unit (RU). Global fitting of the data to a Languir 1:1 model was used to determine the association (K_{on}) dissociation (K_{off}) and affinity constant (K_D) using scrubber2 (Biologic Software Pty Ltd). The experimental Rmax values of integrins β1 and β5 for cANGPTL4 were determined to be 365.6 and 341.9 RU, respectively. The affinity constants of the 6 mAbs for ANGPTL4 were determined using the one shot Kinetics protocol as described by manufacturer (Bio-Rad).

[00115] Purified fibrinogen-like fragment of ANGPTL4 (cANGPTL4) was immobilized onto ProteOn GLC chip by amine coupling, as recommended by the manufacturer (Bio-Rad). Different concentrations of integrins were introduced into the GLC chip at a flow rate of 25 μl/min for 5 min with running buffer (50 mM Tris, pH 8.0, 100 mM NaCl). Polyclonal anti-cANGPTL4 antibodies against the immobilized cANGPTL4 determined the Rmax value to be 423.1 resonance unit (RU). Global fitting of the data to a Langmuir 1:1 model was used to determine the association (k_{on}), dissociation (k_{off}) and affinity constant (K_D) using Scrubber2 (BioLogic Software Pte Ltd). The experimental Rmax values of integrins β1 and β5 for cANGPTL4 were determined to be 365.6 and 341.9 RU, respectively. The affinity constants of the 6 mAbs for ANGPTL4 were determined using the One-Shot Kinetics protocol as described by manufacturer (Bio-Rad).

Detecrtion of Src oxidation by carboxymethylation.

[00116] The detection of reduced Src was performed with minor modifications to the method described in (5). Cells were subjected to anoikis as described above. At indicated times, cells were lysed with 500 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X100, 10 μg/ml leupeptin) containing 100 μM of N-(biotinoyl)-N'-(iodoacetyl) ethylenediamine. Lysate was clarified by centrifugation and c-Src was immunoprecipitated using specific anti-c-Src antibodies. Immunocomplexese were resolved by SDS-PAGE and the biotinylated/reduced fraction of Src Kinase was detected with horseradish peroxidase (HRP)-conjagated streptavidin and by chemiluminescence.

[00117] The detection of reduced Src was performed with minor modifications (Giannoni et al., 2009). Cells were subjected to anoikis as described above. At indicated time, cells were lysed and with 500 µl of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 10 µg/ml aprotinin and 10 µg/ml leupeptin) containing 100 µM of *N*-(biotinoyl)-*N*'-(iodoacetyl) ethylenediamine. Lysates were clarified by centrifugation and c-Src was immunoprecipitated using specific anti-c-Src antibodies. Immunocomplexes were resolved by SBS-PAGE and the biotinylated/reduced fraction of Src kinase was detected with horseradish peroxidase (HRP)-conjugated streptavidin and by chemiluminesence.

Intracellular ROS Assay.

[00118] Cells were subjected to anoikis as described above. Five minutes before the end of each inmcubation time, 5-(and 6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate acetyl ester was added to a final concentration of 10 μM. Cells were lysed in 500 μl of RIPA buffer and analysed by fluorescence analysis using a Perkin Elmer Fluorescence Spectrophotometer at excitation of 485 nm and emission of 525 nm.

Laser Capture Microdissection (LCM)

[00119] For LCM samples, epithelial and stromal fractions were microdissectioned from 8-μm-thick sectioned tissues using PALM Microbeam Axio Observer Z1 (Carl Zeiss) separately. LCM tissues were collected into microfuge tubes with opaque AdhesiveCaps (Carl Zeiss). RNA was extracted using Optimum™ FFPE RNA Isolation kit (Ambion) pooled from 8 LCM tissues. 5 ng RNA was subjected to Full Spectrum Complete Transcriptome RNA Amplification kit (System Biosciences) prior to qPCR as previously described (Chong et al., 2009; Goh et al., 2010).

Generation cANGPTL4 and Antibodies

[00120] Recombinant ANGPTL4 proteins were purified from the conditioned medium of stable cANGPTL4-expressing S2 cells by preparative isoelectric membrane electrophoresis as previously described (Goh et al., 2010). Rabbit polyclonal antibodies

against the C-terminal region and N-terminal region of human ANGPTL4 were produced in-house and previously described (Goh et al., 2010). Monoclonal antibodies (mAbs) against human cANGPTL4 (a.a. 186-406) were made according to standard protocols (Committee on Methods of Producing Monoclonal Antibodies et al., 1999). Briefly, mice were immunized with adjuvant conjugated-cAngptl4. The spleen of the mouse was removed and a single cell suspension was prepared. These cells were fused with myeloma cells and cultured in hybridoma selection medium (HAT; Gibco). The fused cells were cultured in microtiter plates with peritoneal macrophages for 48 hours post-fusion (2-4x 10^6 cells/ml). The cultures were maintained in a 5% CO_2 humidified incubator for 7-21 days, and routinely fed with HAT medium. mAbs in medium were first screened using ELISA to identify positive clones. Positive clones were expanded and recloned by a limiting dilution technique to ensure monoclonality. Next, surface plasmon resonance was performed to determine the binding kinetics of mAbs. Global fitting of the data to a Langmuir 1:1 model was used to determine the association (k_{on}), dissociation (k_{off}) and affinity constant (K_D) using Scrubber2 (BioLogic Software Pte Ltd). mAb 11F6C4 was chosen for immunotherapy and other experiments based on its superior k_{on}, k_{off} and K_D values as well as its ability to block interaction between cANGPTL4 and integrins.

***In Vivo* Tumorigenicity Assay**

[00121] BALB/c athymic nude female mice (20-22 g), aged 5-6 weeks, and C57BL/6J female mice (20-25 g) wide-type (WT), aged 6-8 weeks, were purchased from A*STAR Biological Resources Centre (Singapore). C57BL/6J female ANGPTL4 wild type (WT) and ANGPTL4-knockout (KO) mice were used (Koster et al., 2005). All animals were d maintained in pathogen-free conditions. The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC0092), Nanyang Technological University, and all experiments were carried out in strict compliance with their regulations.

[00122] For nude mice experiments, a total of 5×10^5 cells (A-5RT3$_{CTRL}$ or A-5RT3$_{ANGPTL4}$) were injected subcutaneously (*s.c.*) into the interscapular region of each nude mouse (n = 5 for each group). Injection site was rotated to avoid site bias. The injected tumor cells were allowed to grow for eight weeks. The subcutaneous xenograft tumors were measured externally with a vernier caliper every other day, and tumor volume was estimated by using the equation, $V = (L \times W^2)/2$, where L is the length of the major axis of the tumor, and W is the length of the minor axis. Mice were sacrificed at the end of the experiment (week 8), and their tumors were harvested for further analyses. To test the effect of the number of injected cells on tumorigencity, 0.5x, 2x and $8x10^{-6}$ A-5RT3$_{CTRL}$ or A-

$5RT3_{ANGPTL4}$ were inoculated into nude mice (n = 5) as above. Experiments were terminated at week 4, as tumor volume on $8x10^{-6}$ inoculated group approached 3000 mm^3, accordingly to IACUC protocol. For the antibody treatment, nude mice (n = 6 for each group) were implanted with A-5RT3 as described above. One week post implantation, 30 mg/kg/week of either mAb11F6C4 or isotype control IgG were intravenously (*i.v.*) administrated once weekly for four weeks. The dose of antibody and delivery mode was consistent with studies using mAb14D12, another anti-ANGPTL4 mAb27 (Desai et al., 2007). Mice were sacrificed after treatments, and tumors were harvested for further analyses.

[00123] KO mice and cANGPTL-treated C57BL/6J mice studies were performed as previously described (Sun and Lodish, 2010). Briefly, 1×10^6 $B16F10_{CTRL}$ (scrambled control cells) or $B16F10_{ANGPTL4}$ (ANGPTL4 knockdown cells) were *s.c.* injected into the interscapular region of indicated mice (n=4-6 each group). Mice were *i.v.* treated with either 3mg/kg of cANGPTL4 or control PBS thrice a week. All the animals were monitored and tumor volume measured as above. Mice were sacrificed at the end of the experiment (day 15), and tumors were harvested for photographed.

***In situ* Proximity Ligation Assay (PLA)**

[00124] DUOLink™ *in situ* PLA (OLink Biosciences) was performed on tumor biopsies or cells as described (Tan et al., 2009). Paired-primary antibodies used in the present study were rabbit anti-p(Y397)FAK and mouse anti-FAK antibodies, rabbit anti-pan-14-3-3 and mouse anti-BAD antibodies, mouse anti-cANGPTL4 with either rabbit anti-β1, β3 or β5 integrin antibodies. As a negative control, PLA was performed by using only anti-FAK, anti-pan-14-3-3 or anti-nANGPTL4 antibodies, respectively. Briefly, sections/cells were fixed with 4% paraformaldehyde for 15 min. The slides were washed twice with PBS, blocked for 1 h at room temperature with 2% BSA in PBS containing 0.1% Triton-X, followed by incubation with indicated antibody pairs overnight at 4 °C. PLA was performed as recommended by the manufacturer (OLink Biosciences). Images were taken using LSM710 META confocal laser scanning microscope with a Plan-Apochromat 63x/1.40 Oil objective and ZEN 2008 software (Carl Zeiss).

Electron Paramagnetic Resonance (EPR) Measurement of O_2^-

[00125] Entire excised tumor biopsies were dispersed enzymatically into single cell suspensions. The tissue was minced and incubated in digestion buffer containing hyaluronidase (1 mg/ml), collegenase D (1 mg/ml) and DNase (100 unit/ml) (Sigma-Aldrich) in a 37 °C shaking incubator for 2 h. The dispase and hyaluronidase digests were pooled and filtered through a 70 μm Nylon cell strainer. Cells were washed, pelleted and resuspended in

PBS containing 3% FBS. Equal cell number was used for EPR measurement of O_2^-. Direct trapping of superoxide in aqueous media was performed using the spin trap DEPMPO, which forms a relatively stable superoxide adduct. EPR spectra were recorded at room temperature with a Bruker D-200 ER spectrometer, operating at X-band with a TM 110 cavity with a quartz flat cell. The EPR parameters were set at 100 KHz, X-band microwave frequency, 9.5 GHz; microwave power, 20 mW; modulation amplitude, 1 G; time constant, 160 s; scan time, 50 s; and receiver gain, 5 x 10^5. The EPR spectra represent the averaged signals of 10 scans. EPR signal amplitude at 3480 G represents the pure line, corresponding only to the superoxide adduct. All experiments were performed three times.

Measurement of O_2^- and H_2O_2

[00126] Production of O_2^- from tumor cells were measured using an O_2^--sensitive luciferin derivative, 2-methyl-6-(p-methoxyphenyl)-3, 7-dihydroimidazo[1, 2-a]pyrazin-3-one (MCLA; Invitrogen). 5 x 10^4 of cells were trypsinized, washed, lysed in Krebs buffer and treated either individually or combinatorially for 0.5 h with the following chemicals: superoxide scavenger Tiron (10 mM), NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI, 20 μM) or apocynin (500 μM), mitochondrial complex I inhibitor rotenone (50 μM) and monoclonal human anti-cANGPTL4 anitbody mAb11F6C4 (3 or 6 μg/ml). MCLA (2 μM) was added, and the luminescent signal was recorded immediately thereafter for 1 min with a GloMax® 20/20 Luminometer (Promega). Intracellular H_2O_2 was measured as previously described (Wagner et al., 2005). We performed two control experiments to verify that we were measuring H_2O_2. The specificity of the assay for H_2O_2 was checked with catalase, and the degradation of H_2O_2 or inhibition of the assay system by the sample was checked by determining the recovery of exogenously added H_2O_2. The fold change in O_2^-:H_2O_2 ratio of A-5RT3$_{ANGPTL4}$ and mAb11F6C4-treated tumor cells were determined by direct comparison with the value of either A-5RT3$_{CTRL}$ or control IgG-treated tumor cells, which was arbitrarily assigned the value one.

Caspases Activities Assay

[00127] Cells were subjected to anoikis as described above. The activities of caspases 2, 3, 6, 8 and 9 were measured with Apotarget caspase colorimetric protease assay kit (Biosource International, Camarillo,CA) according to the manufacturer's instructions. O.D.$_{405nm}$ was read and the fold-increase in caspase activities were determined by direct comparison with the level of the A-5RT3$_{CTRL}$ or cognate pre-immune IgG treated cells.

Statistical analysis.

[00128] Statistical significance between two groups was analysed by an unpaired nonparametric test (Mann-Whitney test) or with a student's t-test (SPSS Inc.) All statistical tests were two-sided. P values of ≤ 0.05 were considered significant. Values ($\pm$S.D.) from 3-5 independent experiments with triplicates.

[00129] Statistical significance between two groups was analyzed by an unpaired nonparametric test (Mann-Whitney test) or with a Student's *t*-test (SPSS, Inc.). All statistical tests were two-sided. p value of ≤ 0.05 was considered significant.

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[00130] Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. The invention includes all such variation and modifications. The invention also includes all of the steps, features, formulations and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

[00131] Each document, reference, patent application or patent cited in this text is expressly incorporated herein in their entirety by reference, which means that it should be read and considered by the reader as part of this text. That the document, reference, patent application or patent cited in this text is not repeated in this text is merely for reasons of conciseness.

[00132] Any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention.

[00133] The present invention is not to be limited in scope by any of the specific embodiments described herein. These embodiments are intended for the purpose of exemplification only. Functionally equivalent products, formulations and methods are clearly within the scope of the invention as described herein.

[00134] The invention described herein may include one or more range of values (e.g. size, concentration etc). A range of values will be understood to include all values within the range, including the values defining the range, and values adjacent to the range which lead to

the same or substantially the same outcome as the values immediately adjacent to that value which defines the boundary to the range.

[00135] Throughout this specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. It is also noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of" and "consists essentially of" have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

[00136] Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

We Claims

1. An antagonist to angiopoietin like 4 protein (ANGPTL4) that reduces cell proliferation and reduces the level of Reactive Oxygen Species in a cell, wherein the antagonist comprises an interfering RNA sequence or an antibody specific to angiopoietin like 4 protein (ANGPTL4).

2. The antagonist of claim 1 directed to the C terminal region of the protein.

3. The antagonist of claim 1 or 2 wherein the antibody is a monoclonal antibody and or a humanized antibody.

4. The anatagonist of claim 1 or 2 wherein the interfering RNA sequence comprises that set out in SEQ ID 4, or SEQ ID NO. 5, or SEQ ID NO. 6, or SEQ ID NO. 7.

5. A method of treating a patient to reduce cell proliferation and reduce the level of Reactive Oxygen Species in a cell, which comprises the step of: (a) contacting proliferating cell with an antagonist to angiopoietin like 4 protein (ANGPTL4) wherein the antagonist comprises an interfering RNA sequence or an antibody specific to angiopoietin like 4 protein (ANGPTL4).

6. The method of claim 5 wherein the antagonist is directed to the C terminal region of the protein.

7. The method of claim 5 or 6 wherein the interfering RNA sequence comprises that set out in SEQ ID 4, or SEQ ID NO. 5, or SEQ ID NO. 6, or SEQ ID NO. 7.

8. A method of reducing cell proliferation and reducing the level of Reactive Oxygen Species in a cell, which comprises the step of: (a) contacting proliferating cell with an antagonist to angiopoietin like 4 protein (ANGPTL4) wherein the antagonist comprises an interfering RNA sequence or an antibody specific to angiopoietin like 4 protein (ANGPTL4).

9. The method of claim 8 wherein the antagonist is directed to the C terminal region of the protein.

10. The method of claim 8 or 9 wherein the cell is *in vitro*.

11. The method of claim 8 or 9 wherein the cell is *in vivo*, and the modulator is administered to a subject.

12. The method of claim 8 or 9 wherein the interfering RNA sequence comprises that set out in SEQ ID 4, or SEQ ID NO. 5, or SEQ ID NO. 6, or SEQ ID NO. 7.

13. An antagonist to angiopoietin like 4 protein (ANGPTL4) for the treatment of a cancer wherein the antagonist comprises an interfering RNA sequenceor an antibody specific

to angiopoietin like 4 protein (ANGPTL4) that reduces cell proliferation and reduces the level of Reactive Oxygen Species in a cell.

14. The antagonist of claim 13 directed to the C terminal region of the protein.

15. The antagonist of claim 13 wherein the antibody is a monoclonal antibody and or a humanized antibody.

16. The anatagonist, of claim 13 or 14 wherein the interfering RNA sequence comprises that set out in SEQ ID 4, or SEQ ID NO. 5, or SEQ ID NO. 6, or SEQ ID NO. 7.

17. A composition comprising a therapeutically effective amount of an antagonist to angiopoietin like 4 protein (ANGPTL4) wherein the antagonist comprises an interfering RNA sequence or an antibody specific to angiopoietin like 4 protein (ANGPTL4) that reduces cell proliferation and reduces the level of Reactive Oxygen Species in a cell.

18. The composition of claim 17 wherein the antagonist is directed the C teminal region of the protein.

19. The composition of claim 17 or 18 wherein the wherein the antibody is a monoclonal antibody and or a humanized antibody..

20. The composition of claim 17 or 18 wherein the interfering RNA sequence comprises that set out in SEQ ID 4, or SEQ ID NO. 5, or SEQ ID NO. 6, or SEQ ID NO. 7.

21. The method, antagonist or composition of any one of claims 1 to 20 wherein the antagonist is suitable for use in suppression of tumor growth.

22. The method, antagonist or composition of any one of claims 1 to 20 wherein the antagonist is suitable for use in treating metastasis.

23. A method of diagnosing an anoikis resistance proliferative disorder, comprising the steps of (a) determining an amount of the angiopoietin like 4 protein (ANGPTL4) protein in body fluids or tissue sampled from a person suspected of having a proliferative disorder using an antibody specific to angiopoietin like 4 protein (ANGPTL4) (b) comparing the amount of the angiopoietin like 4 protein (ANGPTL4) from a person suspected of having a proliferative disorder with a second amount sampled from a healthy individual wherein elevated ANGPLT4 in the first sample compared with the second sample is indicative that an anoikis resistance proliferative disorder is present in the person suspected of having a proliferative disorder.

24. The method of claim 23 wherein the antibody is capable of binding selectively a sequence set out in SEQ ID 1, or SEQ ID NO. 2 or SEQ ID NO. 3.

25. The method of claim 23 or 24 wherein the state of tumor progression can be determined by the expression level of the angiopoietin like 4 protein (ANGPTL4) protein in body fluids or tissue sampled increasing from a benign state to a metastatic state.

SEQUENCE LISTING

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