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Highlights

- Elevated expression of ANGPTL4 is a common feature of many human tumor types.
- ANGPTL4 binds integrins to stimulate the NADPH oxidase-dependent production of $\text{O}_2^-$. 
- ANGPTL4 sustains a high $\text{O}_2^-:\text{H}_2\text{O}_2$ ratio to activate pro-survival pathways.
- Suppression of ANGPTL4 impairs tumor growth and enhances anoikis/apoptosis.
Angiopoietin-like 4 protein elevates the pro-survival intracellular O$_2^\cdot$:H$_2$O$_2$ ratio and confers anoikis resistance to tumors

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Running title: ANGPTL4 sustains O$_2^\cdot$ for tumor anoikis resistance.
SUMMARY

Cancer is a leading cause of death worldwide. Tumor cells exploit various signaling pathways to promote their growth and metastasis. The role of angiopoietin-like 4 protein (ANGPTL4) in cancer remains undefined. 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 NADPH oxidase-dependent production of O$_2^\cdot$. A high ratio of O$_2^\cdot$:H$_2$O$_2$ oxidizes/activates Src, triggering the PI3K/PKB$\alpha$ and ERK pro-survival pathways to confer anoikis resistance, thus promoting tumor growth. ANGPTL4 deficiency results in diminished O$_2^\cdot$ production and a reduced O$_2^\cdot$:H$_2$O$_2$ ratio, creating a cellular environment conducive to apoptosis. ANGPTL4 is an important redox player in cancer and a potential therapeutic target.

Significance

We show here that elevated expression of ANGPTL4 is widespread in tumors and tumor-derived ANGPTL4 confers anoikis resistance to tumors via autocrine adhesion mimicry. Our findings that ANGPTL4 hijacks integrin-mediated signaling to maintain an elevated, oncogenic O$_2^\cdot$:H$_2$O$_2$ ratio and therefore confers anoikis resistance to tumor cells suggest ANGPTL4 as an important player in redox-mediated cancer progression. Treating cancer cells with ANGPTL4-targeted RNAi or monoclonal antibodies imparts a significant decrease in in vivo tumor growth and induces apoptosis in cancer cell lines upon anoikis challenge. They suggest that anticancer strategies focusing on redox-based apoptosis induction in tumors are viable.
INTRODUCTION

In response to stresses such as hypoxia and inflammation in the tumor microenvironment, 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 leads to cellular changes including increased proliferation and the ability for cells to grow beyond 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 (i.e., anoikis resistance) is an essential feature of tumor cells. However, the mechanism by which anoikis resistance is acquired remains an unsolved problem in cancer biology.

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; Pervaiz and Clement, 2007). 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 Nox1 (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
metalloproteinases and induce epithelial-mesenchymal transition (Wu, 2006), 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 insights into therapeutic interventions.

The secreted protein angiopoietin-like 4 (ANGPTL4) was recently linked to tumor progression. ANGPTL4 was previously identified as a 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). ANGPTL4 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 but whose function remains unclear. The N-terminal coiled-coil domain of ANGPTL4 (nANGPTL4) mediates ANGPTL4 oligomerization and binds to lipoprotein lipase to modulate lipoprotein metabolism (Ge et al., 2004). Emerging studies also implicate tumor-derived ANGPTL4 in cancer metastasis via its effect on endothelial integrity. However, whether ANGPTL4 promotes or inhibits vascular permeability, and thus cancer metastasis remains controversial. Several previous studies suggest that ANGPTL4 can prevent metastasis by inhibiting vascular leakiness (Galaup et al., 2006; Ito et al., 2003). Conversely, ANGPTL4 is also implicated as a pro-angiogenic factor (Le Jan et al., 2003). Recent reports demonstrate that ANGPTL4 is one of the most highly predictive genes associated with breast cancer metastasis to the lung (Minn et al., 2005; Padua et al., 2008). ANGPTL4 expression is upregulated in clear cell renal-cell carcinoma (Le Jan et al., 2003) and oral tongue squamous cell carcinoma (Wang et al., 2010). In addition, tumor-derived ANGPTL4 has been shown to promote metastasis by disrupting vascular integrity (Padua et al., 2008). The reasons for these conflicting results and the underlying mechanism of
ANGPTL4 activity in tumor cells have not been clarified, hampering our understanding of its precise role in cancer metastasis. More importantly, the global expression pattern of ANGPTL4 in different types of tumors has yet to be fully investigated, and the pathological relevance of ANGPTL4 in cancer biology remains largely undefined. Thus, we set up to study the role of ANGPTL4 in tumor growth and metastasis.
RESULTS

Elevated Expression of ANGPTL4 in Various Tumor Types.

To examine the expression profile of ANGPTL4 in human tumors, we screened its expression pattern on two human tumor tissue arrays, which cover most of the common benign, malignant and metastatic tumors originating from various anatomic sites. Using immunofluorescence (IF) with an anti-cANGPTL4 antibody, we observed widespread, elevated ANGPTL4 expression in all epithelial tumor samples when compared to the corresponding normal tissues, regardless of the anatomical sites of origin (Figures 1A and S1A-B). However, the IF signal level varied among different types of tumors. Notably, the expression of ANGPTL4 increased as tumors progressed from a benign state to an invasive/metastatic state (Figure S1C). Next, we determined ANGPTL4 expression on three human skin tumorigenic lines (HSC, II-4 and A-5RT3), 10 human squamous cell carcinoma (SCC) and 13 basal cell carcinoma (BCC) biopsies by quantitative real-time PCR (qPCR) and immunoblot analyses. Consistent with our prior results, we observed increased ANGPTL4 mRNA and protein levels in these epithelial tumor cells compared with the non-tumorigenic human skin line HaCaT or cognate peri-tumor normal samples (PNSs), respectively (Figures 1B-D). No difference was observed between normal skin biopsies (NS) and PNSs (Figures 1C-D). Interestingly, the three SCCs expressing the highest mRNA level of ANGPTL4 corresponded to an invasive prognosis (Figure 1C), underscoring our finding from tumor tissue arrays. In addition, polyclonal antibodies against either the N- or C-terminus of ANGPTL4 detected only cANGPTL4 in these tumor lines and SSCs (Figures 1B-D and S1D-E). To understand the reason for the increased expression of ANGPTL4 in tumor cells, we examined the expression of hypoxia-inducible factor 1 α (HIF1α) and PPARs in the SCCs. We found a concomitant upregulation of HIF1α with ANGPTL4 in
SSCs than in PNSs (correlation coefficient = 0.88) (Figures 1E and S1F). No clear correlation was observed between the expression of ANGPTL4 and the three PPAR isotypes (Figures S1G-I). These results suggested that at least for SCCs, the elevated ANGPTL4 expression reflects the tumor's hypoxic microenvironment. As a protein that is secreted by tumor cells, ANGPTL4 may perform paracrine or autocrine function in tumors. Therefore, we sought to determine the source of ANGPTL4 in tumors. We isolated epithelial tumor and stromal tissues, the latter consisting mainly of fibroblasts, from SCCs and PNSs, using laser capture microdissection (LCM). qPCR and immunoblot analyses revealed that epithelial tumor cells, rather than tumor stroma, were the major contributor of ANGPTL4 in SCCs (Figure 1F). Further, 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 Tumor Growth.**

Next, we investigated the biological relevance of elevated ANGPTL4 expression to tumor growth via RNAi. Four sets of siRNAs targeting different segments of the ANGPTL4 sequence were stably introduced into the metastatic skin tumor line A-5RT3 (Mueller et al., 2001), and the sub-line with the highest knockdown efficiency (A-5RT3<sub>ANGPTL4</sub>) was selected for subsequent studies. A non-targeting scrambled siRNA was also integrated into A-5RT3 (A-5RT3<sub>CTRL</sub>) as a negative control. ANGPTL4 mRNA and protein levels were suppressed by > 85% in A-5RT3<sub>ANGPTL4</sub> as compared with the parental A-5RT3 or A-5RT3<sub>CTRL</sub> (Figure 2A). The induction of interferon responses has been reported as a challenge to the specificity of some RNAi approaches (Bridge et al., 2003). To test whether the RNAi-mediated silencing of ANGPTL4 was associated with interferon responses, we measured the expression of several key
interferon response genes by qPCR. No induction of $OAS1$, $OAS2$, $MX1$ or $ISGF3\gamma$ was detected in A-5RT3$_{ANGPTL4}$ cells compared with either A-5RT3 or A-5RT3$_{CTRL}$ (Figure S2A).

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 8, but A-5RT3$_{ANGPTL4}$-induced tumors displayed a 90% reduction in tumor growth (Figures 2B-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 S2B). To strengthen the above observations, we subcutaneously implanted B16F10 cells into ANGPTL4-knockout (KO) and control (WT) mice. WT and KO mice were maintained in a C57BL/6J background, and the B16F10 melanoma was derived from the same background. Notably, B16F10 tumor cells implanted in KO mice grew slower than those implanted in WT mice; at day 15, the average tumor volume in KO mice was ~6-fold less than in WT mice (Figure 2D). The injection of ANGPTL4-knockdown (B16F10$_{ANGPTL4}$) cells into KO mice induced little tumor growth, and showed similar growth profile in WT mice compared to control B16F10 (B16F10$_{CTRL}$)-induced tumors in KO mice (Figure 2D). Conversely, WT mice implanted with B16F10$_{CTRL}$ cells and intravenously injected three times a week with recombinant N-terminal histidine-tagged cANGPTL4 showed greater tumor growth. The average tumor volume in cANGPTL4-treated mice was ~3-fold larger than PBS-treated mice (Figures 2E and S2C-D). B16F10$_{ANGPTL4}$-induced tumor growth was diminished in PBS-treated mice as compared to cANGPTL4-treated mice (Figure 2E). Next, we reasoned that treating mice injected with A-5RT3$_{CTRL}$ cells with an antibody that interferes with the action of ANGPTL4 would recapitulate the observation made with A-5RT3$_{ANGPTL4}$ cells. 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 S2E, also see Supplemental Experimental Procedures). Notably, inhibition of ANGPTL4 with mAb11F6C4 attenuated tumor growth in immunodeficient mice, compared with control IgG-treated mice (Figures 2F-G). Immunoblot and IF analyses of A-5RT3\textsubscript{ANGPTL4}\textsuperscript{-}induced tumor biopsies indicated reduced cell proliferation and enhanced cell apoptosis than A-5RT3\textsubscript{CTRL}-induced tumors (Figures 2H-I). A qPCR-focused array of A-5RT3\textsubscript{ANGPTL4}\textsuperscript{-}induced tumor biopsies further suggested increased expression of many pro-apoptotic genes, whereas expression of cell proliferation genes was diminished (Figure S2F; Table S1). Together, these observations clearly support a tumor-promoting role for cANGPTL4.

**ANGPTL4-Deficient Tumor Cells Showed Increased Susceptibility to Anoikis.**

Anchorage-independent growth or anoikis resistance of tumor cells, a hallmark of tumor malignancy (Hanahan and Weinberg, 2000), was investigated by tumor colony formation in soft agar and anoikis assays (Salmon, 1984). Underscoring our *in vivo* findings, the colony-forming potential of A-5RT3\textsubscript{ANGPTL4} cells was undermined and formed fewer (~85%) tumor colonies on soft agar than A-5RT3\textsubscript{CTRL} (Figure 3A). Furthermore, A-5RT3\textsubscript{ANGPTL4} was more susceptible to anoikis, having 30% more apoptotic cells and enhanced caspase activities than A-5RT3\textsubscript{CTRL} cells after 2 h of anoikis (Figure 3B-C). The addition of exogenous recombinant cANGPTL4 reduced the apoptotic index of A-5RT3\textsubscript{ANGPTL4} cells in a dose-dependent manner (Figure 3D). Similarly, ANGPTL4 deficiency in human keratinocytes rendered these cells ~50% more susceptible to anoikis when compared to control keratinocytes, suggesting that a low amount of ANGPTL4 was also necessary to confer anoikis resistance in normal epithelial cells (Figure S3A). No difference
in the apoptotic index was observed due to the deficiency of ANGPTL4 in adhered A-5RT3 cells or keratinocytes (Figure S3B-C).

**ANGPTL4 Interacts with Integrins β1 and β5.**

The mechanism by which ANGPTL4 mediates anoikis resistance is an unanswered question. Previous studies have revealed that anoikis is an integrin-dependent process (Chiarugi, 2008; Zhan et al., 2004). Thus, we hypothesize that ANGPTL4 also exerts its role in tumor cells through integrins-mediated signaling. We examined if cANGPTL4 can interact with integrins. Indeed, SPR and ELISA results showed that ANGPTL4 specifically interacts with integrins β1 and β5, but not with β3 (Figure 3E-F), and these interactions were blocked by either mAb11F6C4 or integrin-specific antibodies (Figures 3G-H and S3D-G). ANGPTL4 deficiency did not affect the expression of integrins β1, β3 and β5 (Figure S3H). An in situ proximity ligation assay (PLA) detected ANGPTL4-integrin complexes in both A-5RT3CTRL cells and tumors (Figures S3I and 3I), confirming that this interaction also exists in vivo. Further investigation revealed that integrin activation by ANGPTL4 binding triggered focal adhesion kinase (FAK) in A-5RT3CTRL cells and tumors, which were reduced by > 70% in A-5RT3ANGPTL4 (Figures S3J and 3J). All of these findings were corroborated by results from immunodetection of FAK on tumor biopsies (Figure 3K). Our findings suggest that ANGPTL4 secreted by epithelial tumor cells acts in an autocrine manner to hijack the integrin/FAK-regulated pathway, conferring anoikis resistance to tumors, and thus sustaining tumor growth.
ANGPTL4 Elevates the \( \text{O}_2^- \) Level and Maintains a High \( \text{O}_2^-:\text{H}_2\text{O}_2 \) Ratio in Tumor Cells.

ROS can be regulated through integrin engagement and an elevated \( \text{O}_2^- \) level allows tumor cells to avoid anoikis (Pani et al., 2009; Pervaiz and Clement, 2007). In this regard, we assessed whether ANGPTL4-integrin interaction regulates 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 decrease in the \( \text{O}_2^- \) level in A-5RT3\text{ANGPTL4} compared to A-5RT3\text{CTRL} cells (Figure 4A-B), suggesting that ANGPTL4 is vital in sustaining \( \text{O}_2^- \) production in tumor cells. To determine the source of \( \text{O}_2^- \), 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 \( \text{O}_2^- \) 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 cellular \( \text{O}_2^- \) level (Figure 4A-B), suggesting that this complex has little role in generating \( \text{O}_2^- \) in tumors. Further excluding mitochondria as the source of ANGPTL4-mediated \( \text{O}_2^- \) generation, qPCR analysis showed no change in the expression of selected genes in the methionine/homocysteine metabolic cycle (Figure S4A), as previously studied in diabetic rodent hepatocytes (Wang et al., 2007). In contrast, the \( \text{O}_2^- \) level was abrogated by using two different NADPH oxidase inhibitors (Ushio-Fukai and Nakamura, 2008), diphenylene iodonium (DPI) and apocynin (Figure 4A-B). ROS 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 which ensures that tumor cells escape from anoikis (Giannoni et al., 2008; Joneson and Bar-Sagi, 1998).

Comparative immunoblot analyses of anti-cANGPTL4 immunoprecipitates from A-5RT3\text{CTRL}- and A-5RT3\text{ANGPTL4}-induced tumor lysates detected integrins \( \beta 1 \) and \( \beta 5 \), along with
phosphorylated FAK and active GTP-bound Rac1, in A-5RT3_CTRL-induced tumors, all of which were reduced in A-5RT3_ANGPTL4-induced tumors (Figure 3K). To further validate the relevance of Rac1 in ANGPTL4-mediated O$_2^-$ production, we transiently transfected A-5RT3_CTRL and A-5RT3_ANGPTL4 cells with dominant-negative Rac1 (T17N) and constitutively active Rac1 (G12V), respectively. We measured a diminished O$_2^-$ level in the former and, conversely, an obviously rescued O$_2^-$ production in the latter. 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 Rac1-engaged Nox (i.e. Nox1-3)-dependent mechanism for O$_2^-$ production. As Nox 3 is expressed predominantly in the inner ear (Paffenholz et al., 2004), we examined the expression of Nox1 and Nox2 in A-5RT3 (Figure S4B). Next, we performed Nox1 and Nox2 knockdown (Nox1 kd and Nox2 kd, respectively) in A-5RT3_CTRL and A-5RT3_ANGPTL4 cells (Figure S4C), and measured the O$_2^-$ level using EPR (Figure 4A-B). Results indicated that Nox1 NADPH oxidase is the predominant source of ANGPTL4-mediated O$_2^-$ generation in tumor cells. The O$_2^-$ level was completely abolished by superoxide scavenger Tiron, which serves as a negative control for superoxide measurements (Figure 4A-B). These data were reproduced by a chemiluminescence assay using 2-methyl-6-(4-methoxyphenyl)-3, 7-dihydroimidazo[1,2-a]pyrazin-3-one hydrochloride (MCLA; Figure 4C) (Münzel et al., 2002). Next, we measured the level of H$_2$O$_2$ 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$_2$O$_2$ levels were higher in A-5RT3_ANGPTL4 than A-5RT3_CTRL cells (Figure 4D). Nox1 knockdown did not affect the H$_2$O$_2$ level, suggesting that ANGPTL4 modulated H$_2$O$_2$ production, linked to an unknown mechanism (Figure S4D). Notably, the lower O$_2^-$ level and O$_2^-$:H$_2$O$_2$ ratio was concurrent with 3-fold more apoptosis and enhanced caspase activities within 2 h of anoikis in A-5RT3_ANGPTL4.
compared to A-5RT3CTRL cells (Figures 4A-D and 3B-C). Accordingly, we observed a reduced O$_2^-$ level in A-5RT3ANGPTL4-induced tumors compared to A-5RT3CTRL-induced tumors (Figure 4E-F), which was associated with increased apoptosis (Figures 2H-I and S2F).

To underscore the relevance of these findings to other cancers, similar experiments were performed using the breast cancer line MDA-MB-231, after using mAb11F6C4 to dose-dependently neutralize endogenous cANGPTL4. We showed earlier that mAb11F6C4 was able to block cANGPTL4-integrin interaction (Figures 3G-H and S3D-G). Consistent with the above results, the inhibition of cANGPTL4 in MDA-MB-231 reduced the O$_2^-$ level (Figure S4E-G), lowered the O$_2^-$:H$_2$O$_2$ ratio (Figure S4H), enhanced apoptosis and caspase activities (Figure S4I-J). Nox1 kd (Figure S4K) but not Nox2 kd reduced ANGPTL4-mediated O$_2^-$ production (Figure S4E-G) with little effect on H$_2$O$_2$ production (Figure S4L). Together, these findings indicate that ANGPTL4 protects tumor cells from anoikis via an NADPH oxidase-dependent O$_2^-$ generation mechanism.

ANGPTL4-mediated O$_2^-$ Activates the Src, PI3K/PKB$\alpha$ and ERK Survival Pathways

Previous reports have shown that ROS produced via integrin engagement oxidizes and activates Src, which stimulates the ERK and PKB$\alpha$ pro-survival pathways (Giannoni et al., 2008; Giannoni et al., 2009; Pani et al., 2009). Both pathways regulate the subcellular localization or stability of BH3-only apoptotic proteins (e.g. Bad and Bim), which are 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. Immunoblot analyses revealed diminished expression of oxidized/activated Src, phosphorylated PKB$\alpha$ and ERK1 in A-5RT3ANGPTL4$^-$induced tumors and A-5RT3ANGPTL4 cells
(Figures 5A and left panel of 5B). Similar immunoblot analyses performed in the presence of DPI and with Nox1 kd cells revealed reduced Src, PKBα and ERK1 activation, emphasizing the role of O$_2^-$ in their activities (Figure 5B). The inhibition of PI3K by LY294002 and Wortmannin, a pivotal upstream mediator of PKBα, caused 4-fold more apoptosis of tumor cells upon anoikis challenge, reaching levels comparable to those of A-5RT3$_{ANGPTL4}$ cells (Figure 5C). In addition, inhibition of MEK1/2, the upstream signal of ERK1, by PD98059 also resulted in an enhancement of apoptotic cell numbers, albeit to a lesser extent (~50%) compared to PI3K inhibitors (Figure 5C). These results suggest that the PI3K/PKBα and ERK1/2 downstream survival pathways are modulated and exploited by ANGPTL4 engagement in tumor cells, the former being the predominant pathway.

The 14-3-3 adaptor protein is known to act downstream of the above 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 was reduced by ~70% in A-5RT3$_{ANGPTL4}$-induced tumors (Figure 5D-F). The Na$^+$/H$^+$ exchanger 1 (NHE), which positively influences cell proliferation by maintaining an alkaline intracellular environment (Akram et al., 2006), was also diminished in A-5RT3$_{ANGPTL4}$-induced tumors (Figure 5D), indicating that NHE plays a subsidiary role in ANGPTL4-mediated tumor cell growth. Upon oxidant challenge in tumor cells, the induction of superoxide dismutase (SOD) expression is muted, allowing tumor cell proliferation (Oberley, 2001; Pervaiz and Clement, 2007). Indeed, we found that cytosolic Zn/CuSOD expression was enhanced in A-5RT3$_{ANGPTL4}$-induced tumors (Figure 5D), which contribute to a reduced O$_2$ :H$_2$O$_2$ ratio via an indirect but linked mechanism (Figure 4D).
ANGPTL4 Deficiency Abrogates O$_2^-$ Production and Sensitizes Cancer Cells to Anoikis

Our results revealed that the suppression of ANGPTL4, either by RNAi (Figure 4A-C) or inhibition with mAb11F6C4 (Figure S4E-G), results in a dose-dependent reduction of O$_2^-$ levels. To underscore the importance of ANGPTL4 in the regulation of O$_2^-$ production, maintenance of a high O$_2^-$:H$_2$O$_2$ ratio, and hence tumor survival, we examined the impact of reduced ANGPTL4 on anoikis in nine different cancer cell lines, in addition to A-5RT3 and MDA-MB-231 cells. Treatment with mAb11F6C4, resulted in a dose-dependent reduction of O$_2^-$ levels (40-80% for 6 µg/ml mAb11F6C4; Figures 6A and S5A), a reduction in the O$_2^-$:H$_2$O$_2$ ratio (70-90% for 6 µg/ml mAb11F6C4; Figures 6B and S5B), a 3- to 8-fold increase in the caspase activities (Figure 7A and S6A) and 30-60% more apoptotic tumor cells (Figures 7B and S6B), all indicating weakened anoikis resistance. A higher percentage of apoptotic tumor cells was also observed using inducible RNAi against ANGPTL4 in the MDA-MB-231 line (Figure S6C). These findings indicate that ANGPTL4-mediated O$_2^-$ production for anoikis resistance may be a common feature in tumor cells. Taken together, our study showed that tumor-secreted ANGPTL4 interacted with integrins in an autocrine fashion to stimulate NADPH oxidase-dependent generation of O$_2^-$, promoting a high O$_2^-$:H$_2$O$_2$ ratio, and consequently activating downstream PI3K/PKBα and ERK activities (Figure 8).
DISCUSSION

The loss of dependence on integrin-mediated ECM contact for growth (i.e. anoikis resistance) is an essential feature of tumor cells, but the mechanism by which anoikis resistance is acquired is a central problem in cancer biology. Our findings demonstrated 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-cell contact. We showed that cANGPTL4 was detected and elevated in many human tumor cells and was predominantly secreted by proliferative tumor epithelial cells. cANGPTL4 specifically binds to integrins β1 and β5 on tumor cells and activates FAK and Rac1, which further stimulates NADPH oxidase-mediated $O_2^-$ production via an autocrine pathway. However, it is conceivable that in tissues/organs expressing high levels of cANGPTL4 in proximity to the tumor site may transmit a paracrine signal. Although integrins alone are not oncogenic, integrin-mediated signalling is often required to enable tumor survival and influence tumor growth (Desgrosellier and Cheresh, 2010). The pro-oxidant intracellular environment led to redox-mediated activation of the Src machinery, and therefore stimulated downstream PI3K/PKBα and ERK pro-survival pathways. This further triggered the 14-3-3 adaptor protein to sequester the pro-apoptotic Bad protein from mitochondria, conferring resistance to anoikis and favouring tumor survival and growth.

The dysregulation of intracellular ROS levels, resulting in an excessive level or persistent elevation of ROS, has been linked to tumor growth, invasiveness and metastasis. Indeed, elevated levels of ROS are detected in almost all cancers (Liou and Storz, 2010). An elevated $O_2^-$ or $O_2^-\cdot H_2O_2$ ratio is particularly important for cancer cells to sustain their tumorigenicity and metastatic potential (Clement and Pervaiz, 2001; Pervaiz and Clement, 2007). We found that 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 NADPH oxidase inhibitors, but not by the mitochondrial complex I inhibitor rotenone. This suggests that $O_2^-$ was “purposely” and enzymatically produced by 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.

The tumor-promoting role of inflammation in the tumor microenvironment is well-recognized (Aggarwal and Gehlot, 2009). PPARγ and δ/β play major roles in the regulation of inflammation and are implicated in tumorigenesis (Peters and Gonzalez, 2009; Murphy and Holder, 2000). Although no correlation between the expression of either PPARγ or δ/β, and their target gene ANGPTL4 was observed in our analysis of PNS and SCCs, we cannot exclude their involvement and/or other oncogenic pathways or cell types in the tumor microenvironment, which 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, PPARβ/δ-deficient fibroblasts can increase the proliferation of normal epithelial cells and SCCs via regulation of the interleukin-1 signaling pathway (Chong et al., 2009). A dysregulated inflammatory response promotes tumorigenesis and malignancy by stimulating ROS production (Aggarwal and Gehlot, 2009). Although not examined in this study, we cannot rule out the possibility that other mechanism to produce $O_2^-$, such as cytosolic 5-lipoxygenase,
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). Despite inconclusive findings from clinical trials on the effect of antioxidants on cancer (Blot et al., 1993; Omenn et al., 1994; Hennekens et al., 1996; Lee et al., 1999), our findings that the specific inhibition of ANGPTL4-mediated integrin signalling and intracellular $O_2^-$ production induce tumor cell apoptosis, suggest anticancer therapeutics focusing on redox-based apoptosis induction remains an exciting and viable strategy.
EXPERIMENTAL PROCEDURES

Human Tumor Samples

Human basal cell carcinoma (BCC) biopsies and squamous cell carcinoma (SCC) biopsies 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. The study was approved by National Healthcare Group Domain-Specific Review Boards (NHG-DSRB). All the tumor samples had been de-identified prior to the analyses.

Tumorigenicity Assay

BALB/c athymic nude female mice (20-22 g), aged 5-6 weeks, and widetype (WT) C57BL/6J female mice (20-25 g), aged 6-8 weeks, were purchased from A*STAR Biological Resources Centre (Singapore). C57BL/6J female WT and ANGPTL4-knockout (KO) mice were used (Koster et al., 2005). The animal studies were approved and carried out in compliance with the regulation from Institutional Animal Care and Use Committee (IACUC0092), NTU. For nude mice experiments, $5 \times 10^5$ cells (A-5RT3_CTRL or A-5RT3 ANGPTL4) were injected subcutaneously (s.c.) into the interscapular region of each nude mouse ($n = 5$). The injection site was rotated to avoid site bias. The injected tumor cells were allowed to grow for 8 weeks. The xenograft tumors were externally measured with a Vernier caliper every other day, and tumor volume was estimated using the equation, $V = (L \times W^2)/2$, where $L$ and $W$ are the length of the major and minor axis of the tumor, respectively. To test the effect of the number of injected cells on tumorigenicity, nude mice were inoculated with $0.5 \times$, $2 \times$ and $8 \times 10^6$ A-5RT3_CTRL or A-
5RT3\textsubscript{ANGPTL4} cells as above. Experiments were terminated at week 4 according to IACUC protocol, because tumor volume in the $8\times10^6$ inoculation group approached 3000 mm\textsuperscript{3}.

For the antibody treatment, nude mice ($n=6$) were implanted with A-5RT3 as above. One week post implantation, 30 mg/kg/week of either mAb11F6C4 or isotype control IgG were intravenously (\textit{i.v.}) administered once weekly for 4 weeks. The dose of antibody and delivery mode was consistent with studies using mAb14D12, another anti-ANGPTL4 mAb27 (Desai et al., 2007). KO mice and cANGPTL-treated C57BL/6J mice studies were performed as previously described (Sun and Lodish, 2010). Briefly, $1\times10^6$ B16F10\textsubscript{CTRL} (scrambled control cells) or B16F10\textsubscript{ANGPTL4} (ANGPTL4 knockdown cells) were \textit{s.c.} injected into the interscapular region of the indicated mice ($n=4-6$). Mice were \textit{i.v.} treated with either 3mg/kg of cANGPTL4 or control PBS three times a week. Animals were monitored and tumor volumes measured as above. Mice were sacrificed at the end of the experiment, and tumors were harvested for further analyses.

\textbf{In situ Proximity Ligation Assay (PLA)}

DUOLink\textsuperscript{TM} \textit{in situ} PLA (OLink Biosciences) was performed on tumor biopsies or cells as described (Tan et al., 2009). The 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, and mouse anti-cANGPTL4 with either rabbit anti-\(\beta\)1, \(\beta\)3 or \(\beta\)5 integrin antibodies. As a negative control, PLA was performed 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 the
indicated antibody pairs overnight at 4 °C. PLA was performed as recommended by the manufacturer. Images were taken using an LSM710 confocal laser scanning microscope with a Plan-Apochromat 63x/1.40 Oil objective and ZEN 2008 software (Carl Zeiss).

**Measurement of O$_2^-$ and H$_2$O$_2$**

Production of O$_2^-$ from tumor cells was measured using an O$_2^-$-sensitive luciferin derivative, 2-methyl-6-(p-methoxyphenyl)-3, 7-dihydroimidazo[1, 2-a]pyrazin-3-one (MCLA; Invitrogen). Cells (5 x 10$^4$) were trypsinized, washed, lysed in Krebs buffer and treated either individually or combinatorially for 0.5 h with the following chemicals: 10 mM Tiron, 20 µM diphenyleneiodonium chloride (DPI), or 500 µM apocynin, 50 µM rotenone and 3 or 6 µg/ml monoclonal human anti-cANGPTL4 antibody mAb11F6C4. 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$_2$O$_2$ was measured as previously described (Wagner et al., 2005). We performed two control experiments to verify that we were measuring H$_2$O$_2$. The specificity of the assay for H$_2$O$_2$ was verified with catalase, and the degradation of H$_2$O$_2$ or inhibition of the assay system by the sample was analyzed by determining the recovery of exogenously added H$_2$O$_2$. The fold change in the O$_2^-$:H$_2$O$_2$ ratio of A-5RT3$_{ANGPTL4}$ and mAb11F6C4-treated tumor cells was determined by direct comparison with the value of either A-5RT3$_{CTRL}$ or control IgG-treated tumor cells, which were arbitrarily assigned the value of one.

**Statistical Analyses**

Statistical significance between two groups was analyzed using unpaired nonparametric test (Mann-Whitney test) or with a Student’s $t$-test (SPSS, Inc.). All statistical tests were two-sided. A p-value of $\leq 0.05$ was considered significant.
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COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.
References


Kersten, S., Mandard, S., Tan, N.S., Escher, P., Metzger, D., Chambon, P., Gonzalez, F.J.,


FIGURE LEGENDS

Figure 1. 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. X, Y and Z axes represent the length, width and immunofluorescence intensity, respectively. Representative images of normal skin and tumor samples with their corresponding heatmaps are shown. Heatmaps from same anatomic sites are grouped horizontally. Results are representative of two independent experiments performed in duplicate. Scale bars represent 200 µm.

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

(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). Normal human skin (NS) biopsies serve as additional controls. Three SSCs with the highest mRNA ANGPTL4 levels corresponded to an invasive prognosis.

(E) Relative HIF1α mRNA and protein levels in paired SCCs and PNSs. For qPCR results, data points from the same individual are linked by colored lines.

(F) Relative ANGPTL4 mRNA and protein levels in laser capture microdissected epithelial cells and stromal fibroblasts from paired SCC and PNS. Hematoxylin and eosin images of an SCC section before and after LCM of epithelial tissue are shown in left panel. Scale bars represent 100 µm. Microdissected tissues were processed for qPCR (middle panel) and immunoblotting (right panel).

(B-F) mRNA data (means ± SD) are from two independent qPCR experiments performed in triplicate. Ribosomal protein L27 (L27) serves as a reference housekeeping gene. *p < 0.05;
**p<0.01; ***p<0.001. Immunoblot data are from three independent experiments performed in duplicate. β-tubulin serves as a loading and transfer control. See also Figure S1.

**Figure 2. Suppression of ANGPTL4 Impairs Tumorigenicity.**

(A) Relative ANGPTL4 mRNA and protein levels in A-5RT3 (parental), A-5RT3CTRL (scrambled control) and A-5RT3ANGPTL4 (knockdown) cells. Data (means ± SD) are from three independent qPCR experiments performed in triplicate. Ribosomal protein L27 (L27) serves as a reference housekeeping gene. Immunoblot data are from three independent experiments performed in duplicate. β-tubulin serves as a loading and transfer control.

(B) Size of xenograft tumors induced in nude mice by 5×10^5 of A-5RT3ANGPTL4 or A-5RT3CTRL cells 8 weeks post-inoculation (n= 5 per group). Each circle represents mean size from three measurements on each mouse at week 8 (wk 8).

(C) Representative pictures of A-5RT3CTRL- and A-5RT3ANGPTL4-induced tumors (wk 8) in (B). Black arrows indicate inoculation sites.

(D-E) 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 (B16F10CTRL, control) and ANGPTL4-knockdown (B16F10ANGPTL4). Cells (1×10^6) were s.c. inoculated into each mouse (n=6 per group). Mice (E) were treated i.v. with either 3 mg/kg of cANGPTL4 or vehicle PBS thrice a week. Values (means ± SEM) are from three measurements of each mouse.

(F) Tumor volume in nude mice injected s.c. with 5×10^5 of A-5RT3 cells and treated i.v. with 30 mg/kg/week of either mAb11F6C4 or control IgG as a function of time (n=6 per group). Each circle represents mean ± SEM from three measurements of each mouse.
(G) Representative pictures of control IgG- or mAb11F6C4-treated nude mice (wk 8) as described in (F). White arrows indicate inoculation sites.

(H) Immunoblot of proliferation (PCNA and cyclin D1), and apoptosis (cleaved caspase-3, Bax and cleaved PARP) markers in A-5RT3ANGPTL4- and A-5RT3CTRL-induced tumor biopsies. Immunoblot data are from three independent experiments performed in duplicate. β-tubulin serves as a loading and transfer control.

(I) Hematoxylin and eosin (H&E) and immunofluorescence staining of A-5RT3CTRL- and A-5RT3ANGPTL4-induced tumor sections. Proliferating (Ki67) and apoptotic (cleaved caspase-3 or TUNEL) cells were identified using the indicated antibodies or assay. Sections were counterstained with DAPI (blue). Scale bars represent 40 μm.

(H-I) All experiments were performed using tumor biopsies harvested from mice described in (B-C) at week 8 (wk 8). See also Figure S2 and Table S1.

*p < 0.05; **p<0.01; ***p<0.00; n.s. denotes not significant.

Figure 3. ANGPTL4 Interacts with Integrins β1 and β5 to Confer Tumor Cells Anoikis Resistance.

(A) Quantification of A-5RT3CTRL and A-5RT3ANGPTL4 tumor colonies on soft agar (left panel). Values (means ± SD) are from four independent assays performed in triplicate. **p < 0.01.

(B) Percentage of apoptotic A-5RT3CTRL and A-5RT3ANGPT4 cells 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-5RT3ANGPT4 cells compared to A-5RT3CTRL cells (assigned value of one) after 2 h of anoikis. Values (means ± SD) are from three independent experiments performed in triplicate. *p < 0.05, **p < 0.01.

(D) Percentage of anoikis-induced apoptotic A-5RT3ANGPT4 cells in the presence of increasing exogenous recombinant cANGPTL4, as analyzed by FACS (5000 events). Vehicle (PBS)-treated A-5RT3CTRL and A-5RT3ANGPT4 cells served as controls for comparison. The apoptotic index is described in (B).

(E-F) Representative sensorgrams of three independent experiments showing binding profiles between immobilized-ANGPTL4 and integrin β1 (E) or integrin β5 (F). Integrin β3 (75 nM) did not show any detectable interaction (F, dotted red line). Sensorgrams were 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.

(G-H) Representative sensorgrams showing dose-dependent blocking of integrin β1 (G) and integrin β5 (H) to immobilized-ANGPTL4 by pre-injection with the indicated concentrations of mAb11F6C4.

(I-J) **In situ** PLA detection of ANGPTL4:integrin β1 (I, left two panels), ANGPTL4:integrin β5 (I, right two panels), and phosphorylated FAK (J) in A-5RT3ANGPT4- and A-5RT3CTRL-induced tumor biopsies. Higher magnification images are shown (I, 2nd and 4th panels; J, right panel).

PLA signals are shown in red and nuclei are stained blue by Hoechst dye. 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 the indicated tumor sections. A configuration-specific monoclonal anti-Rac-GTP antibody was
used for immunoprecipitation of GTP-Rac1. Total FAK serves as a loading and transfer control. Experiments in (I-K) were performed using tumor biopsies described in Figures 2B-C. All experiments in (B-K) were repeated three times with consistent results. See also Figure S3.

Figure 4. ANGPTL4 Elevates $O_2^-$ Level and Maintains a Relatively High $O_2^-:H_2O_2$ Ratio in Tumor Cells.

(A and E) Representative EPR spectra of DEPMPO-superoxide spin adduct from A-5RT3CTRL and A-5RT3ANGPTL4 cells (A) or A-5RT3CTRL- and A-5RT3ANGPTL4-induced tumors (E) in the absence or presence of indicated chemicals or inhibitors. A-5RT3CTRL and A-5RT3ANGPTL4 cells were transiently transfected either with vector expressing Rac1(T17N) or Rac1(G12V), or with ON-TARGETplus siRNA against either Nox1 (Nox1 kd) or Nox2 (Nox2 kd). The superoxide adduct of DEPMPO has hyperfine splitting constants of $a_N$=13.13 G; $a_P$=55.61 G; $a^\beta_H$=13.11 G; $a^\gamma_H$=0.71, 0.42, 0.7, 0.25, and 0.6 G.

(B and F) EPR signal intensity at 3480 G from A-5RT3CTRL and A-5RT3ANGPTL4 cells in (A) or tumors in (E). Tiron-treated measurements serve as negative signal controls.

(C) Measurement of $O_2^-$ levels using the MCLA assay in A-5RT3CTRL and A-5RT3ANGPTL4 cells in the absence or presence of the indicated chemicals or inhibitors.

(D) Measurement of $H_2O_2$ levels using the Amplex red assay in A-5RT3CTRL and A-5RT3ANGPTL4 cells. Arbitrary relative $O_2^--H_2O_2$ ratios are shown in boxes.

(B-D and F) Values were normalized to total proteins and presented as means ± SEM. Data are from three independent experiments performed in triplicate. *p < 0.05; **p < 0.01; ***p < 0.001; n.s. represents not significant. Vehicle-treated A-5RT3CTRL cells (B and C) and A-5RT3CTRL-induced tumor (F) serve as cognate controls. See also Figure S4.
Figure 5. ANGPTL4-mediated $O_2^-$ Regulates Src and Promotes the PI3K/PKBα and ERK Survival Pathways.

(A and D) Immunoblot of the indicated proteins in A-5RT3$_{\text{ANGPTL4}}$ and A-5RT3$_{\text{CTRL}}$-induced tumor biopsies. Values are mean from four independent experiments. c-Src (A) and β-tubulin (D) serve as loading and transfer controls, respectively.

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

(C) Percentage of apoptotic A-5RT3$_{\text{ANGPTL4}}$ and A-5RT3$_{\text{CTRL}}$ 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). Apoptotic index as described in Figure 3B. Sum of Annexin V$^+$/PI$^-$ and Annexin V$^+$/PI$^+$ cells were considered apoptotic. Values are mean from three independent experiments.

(E) In situ PLA detection of 14-3-3:Bad complexes in indicated tumor sections and cells. PLA signals are red dots and Hoechst stained nuclei are in blue. Cells were counterstained with Alexa488-phalloidin for actin stress fibers (green). Negative controls were performed with only
anti-14-3-3 antibodies. Data shown are representative of three independent experiments. Scale bars represent 40 μm.

(F) Number (mean ± SD) of 14-3-3:Bad complexes (E, right panel) was calculated from 200 cells (n = 3; 600 cells total) using BlobFinder software. ***p < 0.001.

**Figure 6. ANGPTL4 Maintains a Relatively High O$_2$⁻:H$_2$O$_2$ Ratio In Tumor Cells.**

Measurement of O$_2$⁻ (A) and H$_2$O$_2$ (B) levels in three different tumor lines by MCLA assay and Amplex red assay, respectively. H$_2$O$_2$ was measured in the presence of the specific catalase inhibitor, 3-amino-1, 2, 4-triazole. Arbitrary relative O$_2$⁻:H$_2$O$_2$ ratios (B) are shown in boxes. Values (mean ± SD) are normalized to the total protein content. Three independent experiments were performed with consistent results. *p < 0.05; **p < 0.01. See also Figure S5.

**Figure 7. Deficiency of ANGPTL4 Activates Caspase Activities and Induces Apoptosis Upon Anoikis in Tumor Cells.**

(A) Relative activities of caspases 2, 3, 6, 8 and 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 of cells treated with pre-immune IgG (6 μg/ml). Values (mean ± SD) are from three independent experiments with consistent results. *p < 0.05; **p < 0.01.

(B) Percentage of apoptotic cells in three tumor lines after 2 h of anoikis as analyzed by FACS (5000 events). Tumor cells were treated with 10 μg/ml of control IgG or mAb11F6C4. Apoptotic index is as described in Figure 3B. Results are mean from three independent experiments. p < 0.05. See also Figure S6.
Figure 8. ANGPTL4-mediated Regulation of O$_2^-$ Production in Tumors.

In an autocrine manner, tumor-derived ANGPTL4 specifically binds to integrins β1 or β5 and subsequently activates FAK and Rac1 activities, which further activates the NADPH oxidase-dependent generation of “onco-ROS” O$_2^-$, promoting a relatively high O$_2^-$:H$_2$O$_2$ ratio in tumor cells. This pro-oxidant intracellular milieu, which may subsidiarily maintained through NHE, favors 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 pro-apoptotic Bad from mitochondria to prevent apoptosis and favor cell survival.
Figure S1, related to Figure 1. Elevated Expression of C-terminal ANGPTL4 (cANGPTL4) in Tumors.

(A and B) Hematoxylin and eosin (H&E) image (A) and immunofluorescence image probed with an anti-cANGPTL4 antibody (B) on melanoma tumor tissue (representative of the tumor tissue array shown in Figure 1A). Higher magnification pictures on areas 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, left upper panel) based on the gray scale value (immunofluorescence intensity) of cANGPTL4 as described in Figure 1A. Scale bars represent 200 µm.

(C) Average integrated gray scale value (immunofluorescence intensity) of cANGPTL4 from various normal and tumor tissues (also see Figure 1A). Tissues from same anatomic site were grouped and compared. A.U.: arbitrary unit. 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 an 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 a 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 with ANGPTL4 mRNA levels were concomitantly up-regulated in SSCs when compared with PNSs (correlation coefficient = 0.88).
(G-I) Relative mRNA expressions PPARα (G), PPARδ (H) and PPARγ (I) in paired human SCCs and PNSs as determined by qPCR. Data spots from same individual are linked by coloured lines. Data (means ± SD) are from two independent qPCR experiments performed in triplicate. Ribosomal protein L27 (L27) was used as a reference housekeeping gene. n.s. represents not significant in the comparison between paired SCCs and PNSs.
Figure S2, related to Figure 2. Suppression of ANGPTL4 Reduces Tumorigenicity and Exogenously Infused cANGPTL4 Accelerates Tumor Growth.

(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-5RT3CTRL (scrambled control cell) and A-5RT3ANGPTL4 (ANGPTL4 knockdown cell). Results (mean ± SD) are from three independent qPCR experiments performed in triplicate. Ribosomal protein L27 (L27) was used as a reference housekeeping gene. n.s. represents not significant in the
comparisons between A-5RT3 and A-5RT3\textsubscript{ANGPTL4} cells or between A-5RT3\textsubscript{CTRL} and A-5RT3\textsubscript{ANGPTL4}.

(B) Mean size of xenograft tumors induced in nude mice by 0.5×, 2× and 8×10\textsuperscript{6} A-5RT3\textsubscript{ANGPTL4} or A-5RT3\textsubscript{CTRL} cells 4 weeks post-inoculation (per group). Values (mean ± SEM) are calculated from n = 5 (per 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 3 mg/kg of cANGPTL4 or control PBS three times a week and dissected 15 days after injection (scale bar 10 mm).

(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 2E) were used. Coomassie stained blot served as loading and transfer control. Experiments were repeated three times with consistent results.

(E) ANGPTL4 interaction kinetic maps for human mAbs, shown as association and dissociation rate constants (\(k_{on}\) 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 SPR. Labels in maps identify the six mAb clones. mAb11F6C4 was chosen for immunotherapy experiment based on its superior \(k_{on}\), \(k_{off}\) and \(K_D\) values.

(F) Heatmap showing genes up- and down-regulated in A-5RT3\textsubscript{ANGPTL4}-induced tumors relative to A-5RT3\textsubscript{CTRL}-induced tumors as determined by qPCR. Results were generated from three pairs of indicated tumors. Experiments were performed using tumor biopsies harvested from mice described in Figures 2B-C at week 8. Three independent qPCR experiments performed in triplicate. Ribosomal protein L27 (L27) was used as a reference housekeeping gene. A detailed description of the genes and their expression is presented in Table S1.
Table S1, related to Figure 2. Relative Fold Change of Gene Expressions in A-5RT3<sub>ANGPTL4</sub>-induced Tumors as Compared with that of A-5RT3<sub>CTRL</sub>-induced Tumors.

Note: The gene expression levels in A-5RT3<sub>CTRL</sub>-induced tumors are assigned value one.
Figure S3, related to Figure 3. ANGPTL4 Effects on Keratinocytes and Its Interaction with Integrins to Activate FAK.

(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 3B.

(B-C) Apoptotic index of adhered epithelial cells. A-5RT3CTRL and A-5RT3ANGPT4 cells (B), and normal 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− (early apoptosis) and Annexin V+/PI+ (late apoptosis) cells were considered apoptotic. Values (bold) denote apoptotic cells (%). Results are representative of three independent experiments.

(D-G) Dose-dependent ANGPTL4 bindings to immobilized integrin \(\alpha\nu\beta5\) (D and E) and integrin \(\alpha5\beta1\) (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 \(\beta1\), \(\beta5\) and \(\beta3\) between A-5RT3CTRL and A-5RT3ANGPTLA cells.

(I-J) In situ PLA detection of ANGPTL4:integrin \(\beta1\) and ANGPTL4:integrin \(\beta5\) complexes (I), and of phosphorylated FAK (J) in A-5RT3CTRL and A-5RT3ANGPTLA cells. PLA signals are shown in red and nuclei are stained blue by Hoechst dye. The cells were also counterstained with Alexa488-phalloidin 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 confocal laser scanning microscope. Scale bars represent 40 \(\mu\)m. PLA images are representative of three independent experiments. Graph (J, right panel) showed mean number of
phosphorylated FAK calculated from 200 A-5RT3\textsubscript{ANGPTL4} and A-5RT3\textsubscript{CTRL} cells (n = 3; total 600 cells) using BlobFinder software (Uppsala University). Error bars represent SD. *p < 0.05. All experiments were performed three or four times with consistent results.
Figure S4, related to Figure 4. ANGPTL4 Elevates the O$_2^-$ Level and Maintains a Relatively High O$_2^-$:H$_2$O$_2$ Ratio in Tumor Cells.

(A) Suppression of ANGPTL4 has no effect in the methionine/homocysteine metabolic cycle of tumor cells. Relative mRNA level of BHMT, MATLA, AHCY, KHK, OAT and HACL1 (representative genes in the methionine/homocysteine metabolic cycle) in A-5RT3$_{ANGPTL4}$ and A-5RT3$_{CTRL}$ cells 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 a loading and transfer control. Representative blots of three independent experiments are shown.

(C and K) Relative fold change in 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), or in MDA-MB-231$_{CTRL}$ (scrambled control), MDA-MB-231$_{Nox1}$ (Nox1 knockdown) and MDA-MB-231$_{Nox2}$ (Nox2 knockdown) cells (K).
(D, H and L) Measurement of H$_2$O$_2$ levels using the Amplex red assay in A-5RT3CTRL and A-5RT3Nox1 cells (D), in MDA-MB-231CTRL and MDA-MB-231Nox1 cells (L); and in MDA-MB-231 cells treated with mAb11F6C4 (3 or 6 µg/ml) or control IgG (6 µg/ml) (H). Arbitrary relative O$_2^-$:H$_2$O$_2$ ratios are shown in boxes (J).

(E) Representative EPR spectra of DEPMPO-superoxide spin adduct from MDA-MB-231 cells 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). In indicated experiments, MDA-MB-231 cells were transiently transfected with ON-TARGETplus siRNA (Dharmacon) against either Nox1 (Nox1 kd) or Nox2 (Nox2 kd). The superoxide adduct of DEPMPO has hyperfine splitting constants of $a_N$=13.13 G; $a_F$=55.61 G; $a_{H}^{\beta}$=13.11 G; $a_{H}^{\gamma}$=0.71, 0.42, 0.7, 0.25, and 0.6 G.

(F) EPR signal intensity at 3480 G from MDA-MB-231 cells in (E). Tiron-treated measurement served as a negative signal control.

(G) Measurement of O$_2^-$ levels using the MCLA assay in MDA-MB-231 cells treated with mAb11F6C4 (3 or 6 µg/ml) or control IgG (6 µg/ml) in the absence or presence of the indicated chemicals or inhibitors.

(I) Percentage of apoptotic MDA-MB-231 after 2 h of anoikis as analyzed by FACS (5000 events). Apoptotic index is described in Figure 3B. Values (bold) denote apoptotic cells (%) from three independent experiments.

(J) Relative activities of caspases 2, 3, 6, 8 and 9 in mAb11F6C4-treated MDA-MB-231 cells after 2 h of anoikis. Values (means ± SD) are from three independent experiments performed in triplicate. *p < 0.05; **p < 0.01. Fold-increase in caspase activity was calculated by comparison to pre-immune IgG-treated MDA-MB-231 cells.
(A, C and K) Error bars represent SD from three independent qPCR experiments performed in triplicate. Ribosomal protein L27 (L27) was used as a reference housekeeping gene. (D-H and L) Values were normalized to total proteins and presented as means ± SEM. Data were from three independent experiments performed in triplicate. *p < 0.05; **p < 0.01; ***p < 0.001; n.s. represents not significant. (E-G) Vehicle-treated MDA-MB-231 cells in the presence of control IgG (6 µg/ml) serve as cognate controls.
Figure S5, related to Figure 6. ANGPTL4 Maintains a Relatively High O$_2$⁻:H$_2$O$_2$ Ratio In Tumor Cells.

Measurement of O$_2$⁻ (A) and H$_2$O$_2$ (B) levels in six different tumor lines by MCLA assay and Amplex red assay, respectively. H$_2$O$_2$ was measured in the presence of the specific catalase inhibitor, 3-amino-1, 2, 4-triazole. Arbitrary relative O$_2$⁻:H$_2$O$_2$ ratios (B) are shown in boxes. Values (mean ± SD) are normalized to the total protein content. Three independent experiments were performed with consistent results. *p < 0.05; **p < 0.01.
Figure S6, related to Figure 7. Deficiency in ANGPTL4 Activates Caspase Activities and Induces Apoptosis in Tumor Cells.

(A) Relative activities of caspases 2, 3, 6, 8 and 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 of cells treated with pre-immune IgG (6 µg/ml). Values (mean ± SD) are from three independent experiments with consistent results.*p < 0.05; **p < 0.01.

(B) Percentage of apoptotic cells in six tumor lines after 2 h of anoikis as analyzed by FACS (5000 events). Tumor cells were treated with 10 µg/ml of control IgG or mAb11F6C4. Apoptotic index is as described in Figure 3B. Results are mean from three independent experiments. p < 0.05. See also Figure S6.

(C) Relative ANGPTL4 mRNA (left panel) and protein (middle panel) levels in MDA-MB-231 cells, whose ANGPTL4 suppression was 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 of treatment. The right panel shows the percentage of apoptotic MDA-MB-231 cells as evaluated by the anoikis assay. Values (mean ± SD) are from three independent experiments with consistent results.
Supplemental Experimental Procedures

Antibodies and Reagents

Antibodies and reagents used in this study: cyclinD1, integrins β1 and β5, (Chemicon); caspase-3, cleaved caspase-3 (R&D Systems); PCNA, β-tubulin, 14-3-3β, 14-3-3σ, Zn/Cu SOD, ERK1, p(T202/Y204)ERK1/2, Nox1, Nox2, secondary HRP-conjugated antibodies (Santa Cruz Biotechnology); c-Src, p(Y416)Src, FAK, p(Y397)FAK, PKBα, p(S473)PKBα, p(T308) PKBα (Cell Signaling Technology); pan-14-3-3 and BAD (Abcam); Na+/H+ exchanger 1 (NHE), Bax and cleaved PARP (Millipore); GTP-Rac1 and total Rac1 (Upstate Biotechnology); Ki67 (NovaCastra); secondary Alexa488-conjugated antibodies, 2-methyl-6-(4-methoxyphenyl)-3, 7-dihydroimidazo[1, 2-a]pyrazin-3-one, hydrochloride (MCLA) and Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen). pFIV lentivirus-based siRNA vector and packaging kit were from System Biosciences. Acetyl ester was from Molecular Probes. Transfection reagent ExGen 500 and restriction enzymes were from Fermentas. Unless specified, all other reagents were obtained from Sigma.

Generation of cANGPTL4 and Antibodies

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., 2010a, 2010b). Rabbit polyclonal antibodies against the C-terminal region and N-terminal region of human ANGPTL4 were produced in-house as previously described (Goh et al., 2010a, 2010b). 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 then 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 h post-fusion (2 - 4 × 10^6 cells/ml). The cultures were maintained in a 5% CO₂ 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, SPR 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.

**Human Tumor Array**

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 the 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 image was automatically stitched using MIRAX Scan software (Carl Zeiss). The 3D heatmaps were
generated using IMARIS software (Bitplane Scientific Software). In the heatmaps, the X- and 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).

**Laser Capture Microdissection (LCM)**

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

**Cell Culture**

HaCaT is an immortalized but non-tumorigenic human keratinocyte line. II-4 and A-5RT3 are tumorigenic HaCaT derivatives kindly provided by the German Cancer Research Center (DKFZ, Germany). HSC is a human squamous cell carcinoma line provided by Prof. Aso (Yamagata University School of Medicine, Japan), and MDA-MB-231 (breast adenocarcinoma) by Dr. Lin (Nanyang Technological University). Other lines used were murine melanoma B16F10 and human tumor lines used were Alexander (malignant hepatoma), A549 (lung carcinoma), Hela (cervix adenocarcinoma), huH-1 (hepatoma), Kato III (stomach signet ring cell carcinoma), MCF7 (breast adenocarcinoma) and T24 (bladder carcinoma). All cells were maintained in
Dulbecco’s modified Eagle’s medium (DMEM; Hyclone, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone), except for A549, huH-1, Kato III which were maintained in RPMI-1640 (Hyclone) with 10% FBS. Cells were cultured at 37 °C, 5% CO₂ and 75% humidified incubator.

**Suppression by RNA Interference (RNAi)**

siRNAs against human ANGPTL4, mouse ANGPTL4, Nox1, Nox 2 and a scrambled sequence as control (control siRNA) were subcloned into the pFIV-H1/U6-puro pFIV/siRNA lentivirus system. The correct pFIV siRNA constructs were verified by sequencing using H1 primer. The sequences are shown in table below. Pseudovirus purification and transduction were performed (Chong et al., 2009). ANGPTL4-knockdown tumor cells were enriched by puromycin selection for 1 week. The A-5RT3 sub-cell line designated A-5RT3\textsubscript{ANGPTL4}, with the highest knockdown efficiency was chosen in this study, and the non-targeted siRNA transduced line was denoted as A-5RT3\textsubscript{CTRL}. The expression of endogenous ANGPTL4 in MDA-MB-231 cells was also suppressed using tetracycline-inducible pSingle-tTS-shRNA vector (Clontech). ANGPTL4 set 2 shRNA sequences were used (see table below). Knockdown efficiency of ANGPTL4 and relative expression level of indicated genes were determined by qPCR and immunoblot.

**Table. Sequences of ANGPTL4, Nox1, Nox2 and Control siRNAs.**

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<tr>
<th>siRNA</th>
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*ANGPTL4* Set 1 siRNA used for lentivirus-mediated RNA interference.

# ANGPTL4 set 2 shRNA was cloned into pSingle-tTS-shRNA vector (Clontech) and used for
doxycycline-inducible knockdown in MDA-MB-231 cells.

**Rho GTPases Assay**

Active GTP-bound Rac1 was quantified as previously described (Tan et al., 2009) with minor modifications. Briefly, 500 μg of the indicated tumor biopsies lysates were incubate with 2 μg of configuration-specific monoclonal anti-Rac1-GTP antibody (GTP-Rac1; NewEast Biosciences). GTP-Rac1-bounded antibodies were immunoprecipitated with Sepharose Protein G/A beads. Bound proteins were solubilised in Laemmli’s buffer, resolved by SDS-PAGE, and immunoblotted using polyclonal antibody against Rac1. Total Rac1 was detected using total lysate. Anti-Rac1 antibodies for immunoblot were from Cytoskeleton Inc.

**Membrane Protein Extraction**

HEK293T cells were transfected with either empty mammalian expression vector pEF1-mycA (Invitrogen) or vector carrying cDNAs encoding human integrins β1, β3 and β5 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 (Tang, 2006). The proteins were dialyzed against PBS prior to SPR analysis.

**Immunoblot Analysis**

Total protein was extracted from cells or tumor tissues/cells with ice-cold lysis buffer (20 mM Na₂H₂PO₄, 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 chemiluminescence (Millipore, USA). β-tubulin was used as loading and transfer control.

**Detection of Src Oxidation by Carboxymethylation**

The detection of reduced Src was performed as described (Giannoni et al., 2009) with minor modifications. Cells were subjected to anoikis as described above. At the indicated time, cells were then lysed with 500 μl 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 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 SDS-PAGE and the biotinylated/reduced fraction of Src kinase was detected with horseradish peroxidase (HRP)-conjugated streptavidin and chemiluminescence.

**Electron Paramagnetic Resonance (EPR) Measurement of O$_2^-$**

Entire excised tumor biopsies were enzymatically dispersed into single cell suspensions. The tissue was minced and incubated in digestion buffer containing 1 mg/ml hyaluronidase, 1 mg/ml collagenase D and 100 unit/ml DNase (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 numbers of cells were 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, $5 \times 10^5$. 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 in triplicates.

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

Total RNA was extracted and qPCR was performed. Expression was related to the housekeeping gene 60S ribosomal protein L27 ($L27$) which did not change under any of the experimental conditions studied. The sequence of primers is available in the table below. For focused mRNA array, genes whose expression was changed significantly (> 2-fold) were listed and heatmaps were generated using Orange Canvas 1.0 software.

**Table. Sequences of qPCR Primers.**

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Surface Plasmon Resonance (SPR) Analysis

The 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 and 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 (kon), dissociation (koff) and affinity constants (KD) 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).

Soft Agar and Anoikis Assay

A-5RT3CTRL and A-5RT3ANGPTL4 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 plates, 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 thiazoly blue tetrazolium in PBS after 4 weeks.

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 doxycycline for 24 h to knockdown ANGPTL4 prior anoikis. For rescue experiments, cells were subjected to anoikis in the presence of either the indicated concentrations of exogenous recombinant cANGPTL4 or vehicle (PBS). Cells were harvested at the indicated time points, and analyzed for apoptosis by FACS analysis. The apoptotic indices of attached cells were determined immediately after harvesting with trypsin.

**Caspase Activity Assay**

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. The O.D. 405nm was measured, and the fold increase in caspase activity was determined by direct comparison with the level of the A5RT3CTRL or cognate pre-immune IgG treated cells.


Supplemental References


