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# The Renin Angiotensin System (RAS) mediates bifunctional growth regulation in melanoma and is a novel target for therapeutic intervention

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Abbreviations: AngII: Angiotensin II; AZA: Azacytidine; $AT_1R$: angiotensin receptor type 1; $AT_2R$: angiotensin receptor type 2; BT: Breslow Thickness; GBM: Glioblastoma multiforme; MVP:

Methylation Variable Position; RAS: renin angiotensin system; SSM: superficial spreading melanoma; TSA: Trichostatin A; VGP: vertical growth phase; RGP: radial growth phase.

**Abstract**

Despite emergence of new systemic therapies, metastatic melanoma remains a challenging and often fatal form of skin cancer. The renin-angiotensin system (RAS) is a major physiological regulatory pathway controlling salt-water equilibrium, intravascular volume and blood pressure. Biological effects of the RAS are mediated by the vasoactive hormone angiotensin II (AngII) via two receptor subtypes, AT1R (encoded by AGTR1) and AT2R (encoded by AGTR2). We report decreasing expression and increasing CpG island methylation of AGTR1 in metastatic versus primary melanoma and detection in serum of methylated genomic DNA from the AGTR1 CpG island in metastatic melanoma implying that AGTR1 encodes a tumour suppressor function in melanoma. Consistent with this hypothesis, antagonism of AT1R using losartan or shRNA-mediated knock-down in melanoma cell lines expressing AGTR1 resulted in acquisition of the ability to proliferate in serum-free conditions. Conversely, ectopic expression of AGTR1 in cell lines lacking endogenous expression inhibits proliferation irrespective of the presence of AngII implying a ligand-independent suppressor function for AT1R. Treatment of melanoma cell lines expressing endogenous AT2R with either AngII or the AT2R-selective agonist Y6AII induces proliferation in serum-free conditions whereas the AT2R-specific antagonists PD123319 and EMA401 inhibit melanoma growth and angiogenesis and potentiate inhibitors of BRAF and MEK in cells with BRAF V600 mutations. Our results demonstrate that the RAS has both oncogenic and tumour suppressor functions in melanoma. Pharmacological inhibition of AT2R may provide therapeutic opportunities in melanomas expressing this receptor and AGTR1 CpG island methylation in serum may serve as a novel biomarker of metastatic melanoma.

## Introduction

Stage IV (metastatic) melanoma frequently presents as large volume, multi-focal disease often with central nervous system (CNS) involvement, with at least 40% of melanoma patients developing brain metastases. Recent therapeutic advances have improved outcomes for some patients with advanced melanoma. These include the use of targeted agents such as the BRAF and MEK kinase inhibitors in patients whose melanomas contain mutations in *BRAF* and *NRAS* and immunotherapy with the anti-CTLA4 antibody ipilimumab (1, 2, 3, 4, 5) and anti-PD-1 agents such as nivolumab and pembrolizumab (6, 7). Despite the introduction of these new approaches to clinical practice, many patients continue to have poor outcomes and there remains a clear need for both novel therapeutic strategies as well as serum biomarkers to inform adjuvant or earlier treatment with immunotherapy.
The renin-angiotensin system (RAS) is a key physiological pathway involved in intravascular volume regulation. Angiotensin II (AngII) an octapeptide hormone is the biological effector and key player with important functions in maintenance of salt-water balance, blood pressure control and other fundamental biological pathways. The effects of AngII are mediated through two G protein coupled receptors, the $AT_1R$ (encoded by *AGTR1*) and $AT_2R$ (encoded by *AGTR2*). Activation of the receptors is thought in some systems to have opposing effects (8). Relatively little is known of the involvement of the RAS in cancer. However, a small subset of oestrogen receptor (ER) positive and HER2 negative breast cancers over-express $AT_1R$ and their growth can be partially inhibited by the selective $AT_1R$ antagonist losartan commonly used as an anti-hypertensive agent (9, 10, 11). Consistent with these observations, down-regulation of *AGTR1* by the miRNA miR-410 suppresses growth, invasiveness, angiogenesis and migration of pancreatic cancer cells, suggesting that $AT_1R$ may also have oncogenic properties in pancreatic cancer (12). In melanoma, inhibition of angiogenesis by losartan was observed in a murine model (13) but no studies have been carried out in human melanoma and the associated function of $AT_2R$ signalling remains unexplored.
Previous studies have identified an array of genes subjected to methylation-dependent transcriptional silencing in melanoma. Furthermore, detection of methylated genomic DNA in peripheral blood is an attractive potential source of cancer biomarkers because of the stability of DNA and the relative specificity of methylation for malignant disease (14). The requirement for sensitive and specific biomarkers of recurrent and particularly unresectable/metastatic melanoma has been emphasised by

evidence from recent clinical trials that efficacy of anti-melanoma therapies is superior, with improved clinical outcomes, when such drugs are deployed in low volume disease (15). We previously showed that serum detection of methylated genomic DNA from *TFPI2* is associated with metastatic melanoma (16) and others have also shown the utility of this approach (17, 18). Herein, we explored the involvement of the two principal angiotensin receptors in melanoma and demonstrate the potential to exploit RAS alteration both for therapeutic and diagnostic purposes.

## Results

### Expression of *AGTR1* and *AGTR2* in melanoma cell lines

We analysed the expression of *AGTR1* and *AGTR2* in a panel of melanoma cell lines arising respectively from radial growth phase (RGP; PMWK, WM35, SBCL2), vertical growth phase (VGP; WM902.B (in figure 1A it is WM902.6), SKMEL224, MEL505 and metastatic melanoma (WM266.4, SKMEL2, SKMEL23, SKMEL30, SKMEL147, SKMEL173, SKMEL501, COLO829, A375). *AGTR1* mRNA was detectable in normal human melanocytes and in each of the RGP cell lines, with the highest expression in PMWK but was greatly down regulated in the WM902B VGP cell line (Figure 1A) and undetectable in SKMEL224 and MEL505. In metastatic melanoma cell lines, expression was undetectable in SKMEL23, WM266.4, SKMEL2 and A375M and greatly down-regulated in SKMEL30 and COLO829 (Fig 1A). Although expressed at lower levels than *AGTR1*, *AGTR2* mRNA was nonetheless detectable in most cell lines with the exception of SKMEL2 and SKMEL224 where there was no detectable expression. Highest expression was observed in PMWK, WM35, MEL505, COLO829 and SKMEL23 (Figure 1A). We also determined whether expression of *AGTR1* and *AGTR2* was affected by serum-free conditions. PMWK, SKMEL23 and SKMEL224 cells were grown in serum-free conditions and mRNA was harvested at various time points and analysedfor *AGTR1* and *AGTR2* expression by qPCR. No change in expression of either gene was detected up to 48 hours (data not shown).

### *AGTR1* silencing occurs in melanoma cell lines via CpG island methylation

Expression analysis revealed down-regulation of *AGTR1* in multiple VGP and metastatic melanoma cell lines (Figure 1A). We wished to explore the mechanistic basis for this observation. A CpG island

is located in the 5´ regulatory sequences of *AGTR1* and we performed pyrosequencing to measure methylation in the cell line panel. Representative pyrograms are shown in Figure 1B and methylation density profiles are presented in Figure 1C. In normal melanocytes, each of the 7 analysed methylation variable positions (MVP) was entirely unmethylated (Figure 1C). In contrast, we observed dense methylation in several of the melanoma cell lines (Figure 1C; Table 1). Of note, the majority of cell lines with dense *AGTR1* CpG island methylation were VGP and metastatic melanomas whereas the RGP cell lines PMWK, SBCL2 and WM35 all showed only low level methylation. In general, there was a good correlation between methylation and transcriptional silencing of *AGTR1*. To further confirm that CpG island methylation causes silencing of *AGTR1*, PMWK, SKMEL23 and SKMEL224 cells were grown in the presence or absence of the demethylating agent 5'-azacytidine. 5'-Azacytidine had no effect on *AGTR1*mRNA levels in PMWK cells (CpG island unmethylated) but resulted in increased levels in SKMEL23 and SKMEL224 (CpG island methylated) (Figure 1D). Addition of the HDAC inhibitor Trichostatin A (TSA) caused a further increase in *AGTR1* mRNA levels in SKMEL23 and SKMEL224 (Figure 1D).

**_AGTR1_ is down regulated by CpG island methylation in metastatic melanoma**

Together, the above data imply that $AT_1R$ may have tumour suppressor function in melanoma and suggests that inactivation of *AGTR1* via CpG island methylation occurs as a late event in melanoma. To validate this hypothesis in clinical tumour samples, we tested a series of paired primary and metastatic melanomas together with control benign nevi for *AGTR1* expression and CpG island methylation using qPCR. *AGTR1* was readily detectable using qPCR in control benign nevi. Expression was typically greatly down regulated in metastatic melanomas relative to matched primary and frequently undetectable in the metastasis (Figure 2A). We then tested *AGTR1* CpG island methylation in primary and metastatic melanomas, again using histologically confirmed benign pigmented nevi as controls. Representative pyrograms are shown in Figure 2B. In each of the benign nevi the *AGTR1* CpG island was unmethylated or methylated at a very low level showing that methylation is a feature of malignant melanocytes (N1-N8, Figure 2C). Similarly, in a series of primary melanomas with a Breslow thickness (BT) of 1mm or less which had not metastasised, the CpG island was invariably unmethylated (P1-P10, Figure 2C). In contrast, in primary melanomas from which metastatic disease subsequently developed, there was a clear increase in methylation compared to low-risk melanomas (P11-P16, Figure 2C). Moreover, in 5/6 cases where matched

primary and metastasis was available, methylation increased from the primary to the metastatic lesion (P11-P16 and M11-M16, Figure 2C).

### *AGTR1* CpG island methylation is a serum biomarker of metastatic melanoma

The association of *AGTR1* CpG island methylation with metastatic melanoma prompted us to explore whether methylated genomic DNA from the *AGTR1* CpG island is detectable in peripheral blood samples of patients with melanoma. We analysed a cohort of sera from 63 patients with (13) or without (50) distant metastatic disease and as controls we used sera from healthy individuals. Serum was isolated using a standardised protocol and methylation in the *AGTR1* CpG island analysed using pyrosequencing. Representative methylation profiles indicating the % methylation at each of the 7 MVPs in the analysed fragment are shown in Figure 2D wherein the increased methylation levels present in sera from patients with metastatic disease are clearly evident. Methylated genomic DNA from *AGTR1* was not detected in the serum of healthy controls. However, positivity for detection of methylated *AGTR1* (defined as % methylation of ≥ 5 at 3 or more MVPs) was significantly higher in patients with metastatic disease (bone, lung liver, spleen) than in those without metastases: 7/13 (54%) vs 6/50 (12%), p=0.0058.

### Down-regulation of *AGTR1* with over-expression of *AGTR2* in melanoma CNS metastases

Central nervous system (CNS) metastasis is a common event in melanoma patients. Given the relationship implied between loss of *AGTR1*and development of metastatic melanoma, we were interested therefore to examine the potential involvement of the RAS in CNS metastasis and to determine the expression patterns of *AGTR1* and *AGTR2*. To address this, we generated four novel CNS metastatic melanoma cell lines de novo from tissue obtained at surgical resection of intra-cranial metastases confirmed by histopathology to be metastatic melanoma and expression of *AGTR1* and *AGTR2* was determined by qPCR in early passage cells. *AGTR1* was expressed in one cell line but was undetectable in the other 3 (Figure 2E). In contrast, *AGTR2* was expressed in 3/4 melanoma brain metastasis cell lines but was not expressed in control brain tissue (Figure 2E).

### Antagonism of $AT_1R$ confers serum independence to melanoma cell lines

Transcriptional silencing of *AGTR1* implies a tumour suppressor rather than oncogenic function for *AGTR1* in melanoma. To seek mechanistic evidence to support this hypothesis, we investigated the effect of antagonism of $AT_1R$. Since expression of *AGTR1* was highest in PMWK and SKMEL147 (Figure 1A), we selected these cell lines to initially assess the effect of antagonism using the selective $AT_1R$ blocker losartan. In serum replete conditions, losartan had no effect as both cell lines readily proliferated. However, in serum free conditions an increase in proliferation was observed in both cell lines on day 3 and day 5 in the presence of losartan (Figure 3A and 3B). Promotion of PMWK and SKMEL147 proliferation by losartan was reproducibly observed in multiple independent experiments (PMWK: p=0.0066, SKMEL147: P=0.005). To confirm the specificity of the effect of losartan on proliferation we tested SKMEL224 cells that do not express *AGTR1*. These cells grew readily in serum-free conditions and growth was unaffected by the addition of Losartan in contrast to the clear growth promoting effect of losartan on PMWK cells (Figure 3C). Similarly, losartan had no effect on the proliferation of MEL505 and SKMEL30 that also lack expression of *AGTR1* but promoted serum-free growth of C8161and SKMEL173 both of which express *AGTR1* (data not shown). We conclude, therefore, that the ability of losartan to promote melanoma growth requires expression of *AGTR1*.

**Loss of $AT_1R$ confers growth factor independence in melanoma**

Rather than acting as an oncogenic receptor (as reported in a subset of breast and other cancers), our results imply a negative growth regulatory function for $AT_1R$ in melanoma. To further examine the association between the absence of $AT_1R$ and serum-free growth in melanoma we used inhibitory RNA to modulate expression of *AGTR1*. We generated stable knockdowns using the TRIPZ Inducible Lentiviral shRNA vector system (Dharmacon) that allows reversible, controlled gene silencing. The vector is designed to be Tet-On® such that shRNA expression is induced in the presence of doxycycline. PMWK cells stably expressing the *AGTR1* shRNA vector or the non-silencing control vector were cultured in 10% FBS, 2% FBS and 1% FBS conditions in the presence or absence of doxycycline. Doxycycline was added at 2μg/ml daily for the course of the experiment to maintain *AGTR1* knockdown. Knockdown of *AGTR1* was confirmed by qPCR and western blotting (Figure 4A, upper panels). Proliferation of cells was assessed on day 5. All cells maintained in 10% serum were able to proliferate in the presence or absence of doxycycline (Figure 4A, lower panels). However, in 1% serum conditions, PMWK cells expressing *AGTR1* shRNA grew more

efficiently in the presence of doxycycline (where *AGTR1* is knocked down) than in its absence and more efficiently than the non-silencing cells in both conditions ($p<0.05$; Figure 4B). These data show that even modest decreases in levels of *AGTR1* result in enhanced proliferation in conditions of growth factor limitation.

### *AGTR1* is a ligand independent suppressor of melanoma growth

Next, we examined the effect of ectopic expression of *AGTR1* in cells that lack endogenous expression of the gene. We transfected the *AGTR*1 null cell line SKMEL224 with escalating concentrations of *AGTR1* expression plasmid or control plasmid (0.1μg -1μg) and selected transfectants in G418. Surviving colonies transfected with the control plasmid were obtained at comparable frequency in all control plasmid concentrations but no transfectants were obtained at any of the *AGTR1* expression plasmid input concentrations (Figure 4C). Importantly, the colony suppressing effect of ectopically expressed *AGTR1* did not require exogenous AngII. Further attempts to generate stable *AGTR1* expressing clones in serum and serum-free conditions and using another null line (SKMEL23) were also unsuccessful (Figure 4C). We also performed clonogenic assays by transiently transfecting SKMEL224 and SKMEL23 cells in serum and serum free conditions as described in methods. However, as with our previous experiments, in both serum conditions colonies were only obtained with the control plasmid. To rule out a potential non-specific toxic effect of the *AGTR1* expression plasmid, we transfected the *AGTR1* expressing line, PMWK with this plasmid and successfully generated stable clones (Figure 4C). Taken together, these results imply that ectopic expression of *AGTR1* is detrimental to cell lines lacking expression of the endogenous gene and this effect is independent of AngII.

### AngII drives proliferation of melanoma cell lines expressing $AT_2R$

AngII has been shown to promote proliferation in a subset of breast cancers which overexpress $AT_1R$ and given the evidence of transcriptional silencing of *AGTR1* in melanoma we wished to determine the effect of AngII on melanoma proliferation. Accordingly, we initially tested the effect of varying concentrations of AngII (1pM-100pM) on the proliferation of melanoma cell lines with differing expression of $AT_1R$ and $AT_2R$ (as determined in Figure 1A), namely PMWK (AGTR1 + / AGTR2 +), SKMEL 224 (AGTR1 - / AGTR2 -) and SKMEL23 (AGTR1 - / AGTR2 +). Under normal culture conditions in media supplemented with 10% serum (non-starved) AngII had no effect on the

proliferation of GBM cell lines irrespective of their expression of angiotensin receptors (Figure 4D). Foetal bovine serum (FBS) contains an abundance of growth promoting as well as inhibitory factors that could mask the growth enhancing effects of angiotensin II (19). We therefore repeated this experiment in serum free conditions and under these conditions significant differences in growth were observed (Figure 4D). In serum-free medium and in the absence of AngII, PMWK and SKMEL23 cells proliferated minimally. However, in the presence of AngII both cell lines exhibited a significant dose-dependent increase in growth (PMWK: 1μM, p=0.0896; 10nM, p=0.01; 100pM, p=0.0026; 1pM, p=0.041. SKMEL23: 1μM, p=0.0288; 10nM, p=0.0372; 100pM, p=0.0185; 1pM, p=0.8004). The maximum growth-promoting effect occurred in both cell lines at 100pM AngII (Figure 4D). Essentially similar effects of AngII were also observed in SKMEL505 (AGTR1 - / AGTR2 +). In contrast, AngII had no effect on proliferation of SKMEL224 in serum-free medium (Figure 4D). These results are consistent with the growth-promoting effects of AngII being mediated via $AT_2R$. To test this hypothesis, we determined the effect on melanoma cells of Y6AII which we recently showed to be a highly selective agonist of $AT_2R$ (20). PMWK cells were cultured in serum-free medium in the presence or absence of varying concentrations of Y6AII and proliferation assessed on day 5. As shown in Figure 4E, there was a clear dose-dependent increase in proliferation in cells treated with Y6AII, with 100 μM Y6AII causing an increase in proliferation at levels comparable to stimulation with 100pMAngII (Figure 5B). Y6AII caused an increase in proliferation of PMWK across a range of concentrations and this was maximal at10μM (p=0.048) and100μM (p=0.0003). In contrast, Y6AII had no effect on proliferation of the *AGTR2* negative cell lines SKMEL2 and SKMEL224 (Data no shown).

**Selective blockade of $AT_2R$ inhibits melanoma growth <u>in</u> <u>vitro</u> and <u>in</u> <u>vivo</u>**

These results imply a role for $AT_2R$ rather than $AT_1R$ in promoting melanoma growth and prompted us to determine whether pharmacological inhibition of $AT_2R$ inhibits melanoma growth. We first evaluated the effect of PD123319 a selective, non-peptide $AT_2R$ inhibitor. In the absence of exogenous AngII, PD123319 had no effect on proliferation of any of the tested cell lines in either 10% serum or serum-free conditions (Data not shown). However, PD123319 efficiently blocked the growth promoting effects of AngII in serum free conditions in both PMWK (p=0.05) and SKMEL23 (p=0.0096) (Figure 5A). In SKMEL224, neither AngII nor PD123319 had any effect on proliferation (Figure 5A). Having observed that PD123319 inhibits melanoma cells expressing $AT_2R$, we tested

the more potent EMA401, a highly selective tetrahydroisoquinoline $AT_2R$ antagonist (21). EMA401 inhibited proliferation of PMWK cells, with almost complete inhibition at 30μM in the absence of exogenous AngII (Figure 5B). We then wished to determine whether the ability of AT2R-blocking agents to inhibit melanoma was also seen in vivo. For this, we used a zebra fish model which is recognised as an excellent system in which to study melanoma (23,24). SKMEL23 cells were injected into zebrafish yolksac and the fish grown with or without 30μM EMA401 in their medium. The presence of EMA401 greatly reduced the proliferation of the melanoma cells (Figure 5C). Similar inhibition of proliferation was seen in PMWK cells.

**Selective blockade of $AT_2R$ inhibits melanoma angiogenesis**

AngII signalling is known to be an important determinant of angiogenesis. We therefore sought to determine whether the ability of melanoma cells to promote angiogenesis is affected by selective blockade of $AT_1R$ and $AT_2R$. To assess the role of angiotensin receptor signalling in the induction of tumour associated angiogenesis, we utilised the assay described by Carpentier et al (22). Human microvascular endothelial cells (hCMEC/D3) were exposed to conditioned media (CM) collected from PMWK or SKMEL23 cells treated with angiotensin II alone or in combination with the angiotensin receptor antagonists Losartan, PD123319 and EMA401. The effects on endothelial tube formation (master segments length) and on network integrity (number of meshes) was determined after 24 hours. Exposure of hCMEC/D3 cells to medium conditioned by PMWK or SKMEL23 resulted in an increase in master segments length and tube formation. This was not potentiated by exposure of cells to exogenous AngII during the conditioning phase. However, both losartan and PD123399 blocked the increase brought about by the CM with EMA401 demonstrating superior efficacy (Figure 6A and 6B).

**Selective blockade of $AT_2R$ potentiates anti-BRAF therapy**

We noted that some of the cell lines with low expression of *AGTR1* contained the *BRAF* V600 mutation which confers sensitivity to BRAF and MEK inhibitors (A375, WM239, WM35, COLO829). This led us to evaluate whether inhibition of $AT_2R$ affects response to these agents. We tested this question in A375 and WM329 cell lines which express low to undetectable *AGTR1* and readily detectable *AGTR2* and in two further cell lines (WM164 and WM793) which express

AGTR2 and positive for *BRAF* V600 mutation. Cells were grown in medium containing 1% serum and challenged with various concentrations of dabrafenib with or without EMA401. As observed previously, PD123319 alone had no effect on proliferation of cell lines under these growth conditions (data not shown). However, EMA increased the sensitivity of all the tested cell lines to dabrafenib, implying that inhibition of $AT_2R$ increases cellular sensitivity to targeted agents in melanoma (Figure 6C). This is most clearly illustrated in A375 cells in which dabrafenib and EMA401 individually only incompletely inhibit proliferation but together produce complete inhibition.

## Discussion

The importance of the RAS in regulating intravascular volume is well-recognized and therapeutic manipulation of the RAS is a mainstay of antihypertensive and heart failure management. There is evidence in breast cancer that over-expressed $AT_1R$ has oncogenic function(s) (9, 10, 11), but this is restricted to a subset of oestrogen receptor positive HER2 negative cases in which AngII promotes and losartan inhibits proliferation. In the current work, we report that AngII drives proliferation of melanoma and to the best of our knowledge this is the first demonstration that AngII is mitogenic in human melanoma cells. We also provide several lines of evidence that (in contrast to the situation in breast cancer) $AT_1R$ acts as a tumor suppressor rather than as an oncogenic receptor and, in addition, $AT_2R$ acts as an oncogene in melanoma. Together, the opposing functions of $AT_1R$ and $AT_2R$ reveal that the RAS is a bifunctional growth regulator in melanoma. Fundamental to our investigation of the function(s) of $AT_1R$ and $AT_2R$ in melanoma is the use of highly selective agonists and antagonists to manipulate the endogenous activity of each receptor, rather than cell lines that have been engineered to ectopically over-express either gene. Additional work to investigate the precise mechanism by which $AT_1R$ acts as a tumour suppressor in melanoma is being undertaken using inducible expression in appropriate systems.

We are not aware of previous experimental evidence implying a tumor suppressor function for $AT_1R$ in human malignancy but we now provide multiple, complementary lines of evidence to support the candidacy of *AGTR1* (encoding $AT_1R$) as a tumor suppressor in melanoma. First, antagonism of $AT_1R$ by losartan promotes the growth in serum-free medium of melanoma cell lines that express *AGTR1*. We first observed this effect in PMWK (RGP) melanoma cells, but also subsequently in SKMEL147 (metastatic), C8161 (metastatic) and other melanoma cell lines that express *AGTR1*. The effect of $AT_1R$ blockade in promoting proliferation is thus common to melanoma cell lines expressing *ATGR1*, losartan having no effect in cell lines lacking *AGTR1* due to transcriptional silencing, affirming the specificity of the effect. The ability of losartan to promote melanoma proliferation is in clear contrast to its anti-proliferative effect in breast cancer cells over-expressing $AT_1R$ (9, 10, 11) and to a study in murine melanoma cells (13). To the best of our knowledge, there are no previous reports that pharmacological blockade of $AT_1R$ promotes cancer cell growth. Second, targeted down-regulation of *AGTR1* using inducible shRNA conferred increased ability to

proliferate in serum-free and low serum medium in cell lines expressing *AGTR1* and thereby mimicked the effect of losartan. Again, we are not aware of previous evidence demonstrating this effect. Third, ectopic expression of *AGTR1* in cell lines with transcriptional silencing of the endogenous gene resulted in cell death making it impossible to derive clones stably expressing *AGTR1* from parental cells with no or low endogenous expression. However, no inhibitory effect on proliferation was seen in cell lines that express endogenous *AGTR1*. This effect was reproducibly observed in the absence of exogenous AngII and in both serum replete and serum-free conditions. These observations are consistent with a tumour suppressor function for *AGTR1* that can occur in a ligand-independent manner. We note that ligand-independent activation of $AT_1R$ (and $AT_2R$) is well-recognised (25, 26). Fourth, *AGTR1* expression is silenced by CpG island methylation in melanoma but not in control benign nevi. Transcriptional silencing in neoplastic but not equivalent normal tissue is a recognized feature of tumour suppressor genes and we are not aware of any previous reports of methylation-dependent transcriptional silencing of *AGTR1* in cancer. The frequency with which *AGTR1* is silenced implies strong selective pressure to abrogate the function of $AT_1R$ in advanced melanoma and is consistent with our functional analyses demonstrating that inactivation (either pharmacologically with losartan or by shRNA) confers a growth advantage to melanoma cells in allowing serum-free proliferation and with the proposed function of *AGTR1* as a ligand-independent tumour suppressor. The specificity of *AGTR1* methylation for malignant melanocytes and its association with advanced / metastatic melanoma is also consistent with a tumour suppressor function for $AT_1R$.

Despite the negative growth regulatory effect of *AGTR1*, we show in this study that AngII nonetheless promotes serum-free proliferation in melanoma cell lines and we demonstrate several lines of evidence to support the hypothesis that this effect occurs via $AT_2R$. First, AngII had no mitogenic effect on the serum-free proliferation of cell lines lacking expression of $AT_2R$, as exemplified by comparison of SKMEL 23 and SKMEL224. In both cell lines expression of *AGTR1* is silenced by CpG island methylation whilst $AT_2R$ is expressed in SKMEL23 but not in SKMEL224 and AngII promoted serum-free growth in SKMEL23 but not in SKMEL224. Furthermore, A375 cells which, like SKMEL23, have a silenced *AGTR1* but express *AGTR2* also demonstrate growth promotion in serum-free conditions by AngII. Second, growth of melanoma cells expressing *AGTR2* was blocked by the $AT_2R$ selective antagonists PD123319 and EMA401. Third, Y6AII, a highly selective $AT_2R$ agonist (20) phenocopied the mitogenic effect of AngII but only in melanoma

expressing $AT_2R$. Promotion of proliferation by Y6AII strongly supports our hypothesis that AngII acts as a mitogen in melanoma via $AT_2R$ and not $AT_1R$. Fourth, *AGTR2* is over-expressed in several novel early passage cell lines established de novo in our laboratory from histologically confirmed melanoma CNS metastases. Given the low expression of *AGTR2* in normal melanocytes, the frequent over-expression of *AGTR2* implies that this confers a growth advantage in advanced melanoma. Of note, *AGTR1* was undetectable in the majority of these cell lines, consistent with a model in which silencing of *AGTR1* abrogates the ligand-independent growth suppressor effect of *AGTR1* and over-expression of *AGTR2* promotes proliferation via ligand-dependent mechanisms.

Together these mechanistic studies suggest that loss of $AT_1R$ confers a more aggressive phenotype to a subset of melanomas. Accordingly, the expression of *AGTR1* was greatly decreased in metastatic relative to matched primary clinical melanomas and, conversely, *AGTR1* methylation quantitatively increased in metastatic derivatives of primary clinical melanomas. We propose, therefore, a model in which the status of the RAS influences the biology of melanoma. In early disease, exemplified by the RGP cell line PMWK, *AGTR1* is expressed and the clinical phenotype is typically not aggressive. With malignant progression, *AGTR1* is silenced by CpG island methylation conferring a more aggressive phenotype (for example by allowing a proliferative advantage in low growth factor conditions), whereas *AGTR2* is expressed and acts as the transducing receptor for the mitogenic effects of AngII.

Our data have clear potential clinical implications. First, we have shown that blocking $AT_2R$ with the antagonists PD123319 and EMA401 inhibits proliferation of melanoma cells expressing *AGTR2* in vitro and in vivo in a zebrafish model. The most robust inhibition was seen with EMA401. This agent is a first in class potent and highly selective antagonist of $AT_2R$ and is under clinical development to treat peripheral neuropathic pain (27). Whereas the effect of PD123319 was evident in the presence of exogenous AngII, EMA401 exhibited complete inhibition of melanoma proliferation in the absence of exogenous AngII. Of note, the anti-proliferative effects of $AT_2R$ blockade occurred in both *BRAF* V600 mutation positive, *NRAS* mutation positive and in double negative (*BRAF* & *NRAS* wild-type) cell lines implying that inhibition of $AT_2R$ may be a viable therapeutic strategy in melanomas lacking common driver mutations. We also show that antagonism of $AT_2R$ potentiates the anti-proliferative effect of small molecule BRAF inhibitors. We demonstrate potentiation of dabrafenib in 4 independent *BRAF* V600 mutation positive, $AT_2R$ positive cell lines. Therapeutic synergy without additional toxicity implies that this combination may improve the

clinical utility of currently used anti-melanoma drugs. Studies to determine the optimal combinations of $AT_2R$ blockers with BRAF and MEK inhibitors using in vivo models are in progress. Third, the ability of AngII to drive proliferation of melanoma implies that agents which reduce serum levels of AngII may have growth inhibitory activity. The Angiotensin Converting Enzyme inhibitors (ACEI) such as lisinopril and ramipril, although producing only incomplete inhibition of ACE (28), are widely used in cardiovascular medicine. Later generation agents that inhibit renin mediate substantial reductions in serum AngII levels (29). One such agent, Aliskiren, produces highly efficient reduction in serum AngII and has potent anti-hypertensive properties. It would be of interest to determine the effect of Aliskiren on melanoma growth in vivo. We show in our study that *AGTR2* is over-expressed in early passage cell lines developed in our laboratory from CNS melanoma metastases. Importantly, the use of Aliskiren to deplete serum AngII as a potential therapeutic strategy for CNS melanoma metastasis would abrogate the requirement for passage of the drug across the blood : brain barrier.

Indirect support for the hypothesis that AngII may promote cancer growth and that expression of *AGTR1* may correlate with a better prognosis is afforded by the results of a number of epidemiological studies. Yoon et al (29) demonstrated a generalized decreased risk of cancer associated with use of $AT_1R$ blockers in cohort and nested case-control studies and in studies with long-term follow-up of more than five years. Critically however, in subgroup meta-analysis an increased risk specifically of melanoma and renal cancer was reported in patients using ATR blockers. Of note, *AGTR1* is also subject to transcriptional silencing in metastatic clear cell carcinoma of the kidney (unpublished observations). In a study in lung cancer, low ACE levels were predictive of OS benefit from cediranib (30) and higher activity of the ACE-AngII-AGTR1 axis associated with a better response to bevacizumab in cancer (31).

We have shown in the present study that methylation of *AGTR1* is associated with metastatic melanoma. Furthermore, analysis of a series of patient-matched primary: metastatic melanomas demonstrated that methylation levels in the *AGTR1* CpG island frequently increase in the progression of a primary cutaneous melanoma to a metastatic lesion. This association of *AGTR1* CpG island methylation with metastatic disease was reflected in the presence of methylated genomic DNA in the peripheral blood of a subset of patients with metastatic disease, implying that detection may have utility as a biomarker, particularly of visceral and CNS metastatic disease. We are investigating this

possibility in large, prospectively collected series of sera with known clinical outcome analyzing *AGTR1* both alone and in combination with *TFPI2* (16).

In conclusion, we show here that the status of the RAS is an important determinant of the biological properties of melanoma and may be exploitable both diagnostically and therapeutically.

## Materials and Methods

### Cell lines and drugs

Melanoma cell lines were routinely grown as described previously (16). Primary cultures of brain metastatic melanomas M5, M78, M80 and M88 were established from fresh tumour tissue obtained from neurosurgical resection of intracranial lesions confirmed to be metastatic melanoma by histopathology. Tumours were washed in DMEM: F12 (1:1) (Gibco), minced through a cell strainer (BD Falcon) to obtain a single cell suspension and cells pelleted by centrifugation for 10mins at 12K rpm. The pellets were briefly re-suspended in 1ml of sterile dH20 to lyse contaminating red blood cells followed by the addition of 10ml of DMEM: F12 + 10% FBS to neutralise the effects of dH20. Cells were pelleted as before and cultured in fresh DMEM: F12 +10% FBS at $37^0$C. The selective $AT_1R$ inhibitor losartan and the highly selective $AT_2R$ agonist Y6AII recently developed by us were synthesised as described previously (20). AngII and PD123319 were purchased from Sigma. EMA401, a highly selective tetrahydroisoquinoline AT2R antagonist (21), was kindly provided by Novartis pharmaceuticals. Demethylation experiments using azacytidine and trichostatin were done as described previously (33, 34). Dabrafenib was purchased from Sigma.

### Plasmid vectors and generation of stable clones

To generate an *AGTR1* expression vector, the *AGTR1* ORF was purchased from Origene and sub-cloned into pcDNA3-DEST40 using Invitrogen's Gateway Technology to generate pcDNA3.2/V5/GW/AGTR1. pcDNA3.2/V5/GW/CAT served as the control plasmid. The sequence of both plasmids was verified by sequencing. The TRIPZ AGTR1 shRNA plasmids (set of 5) for inducible knockdown of AGTR1 and the non-silencing control plasmid were purchased from DE Dharmacon. Cells were transfected with 1ug of each of the plasmids using metafectine (Biontex) as

per manufacturer's instructions and transfectants selected in 0.5µg/mL puromycin (Sigma). Expression of the *AGTR1* or non-silencing shRNA was induced by adding 2 µg/mL doxycycline (Sigma) every 48 hours. Transfectants were analysed for knockdown of *AGTR1* by qPCR and by Western blotting using the Novus Biologicals antibody (NBP-77078) at a dilution of 1:250.

**Proliferation analysis**

Cell proliferation was measured using the sulforhodamine b (SRB) colorimetric assay. Cells were seeded into 96 well plates (Costar) at a density of $4x10^3$ cells per well in media containing 10% FCS. 24hrs after plating culture media was replaced either with media containing 10% FBS (non-starved) or 0% FBS (starved). After a further 24hrs hours cells were treated with drugs as appropriate and incubated for various time points (D1, D3, D5) and harvested by fixing in 10% cold trichloroacetic acid (TCA) for at least 1 hour at 4°C. Fixed plates were washed 4 times with $dH_2O$ and air dried at room temperature prior to staining with 60 µL 0.4% SRB for 1h at RT. Unbound SRB was removed by washing plates 4 times using 0.1% acetic acid and allowed to air dry at room temperature. Bound SRB was re-suspended in 150µL 10 mmol/L Tris pH 10.5 and the absorbance read at 490nm using an ELx800 microplate reader (BioTek).

**Clonogenic Assays**

Clonogenic assays using transiently transfected cells were performed by plating $2x10^5$ cells / 6cm dish and transfected after 24h with 1µg of pcDNA3.2/V5/GW/AGTR1 or control vector pcDNA3.2/V5/GW/CAT using Metafectine (Biontex) according to the manufacturer's instructions. Transfection efficiency was 25–30% under these conditions as measured by immunofluorescence staining using V5 antibody. Essentially transfected cells were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.25% Triton™ X-100 for 10 minutes, and blocked with 5% BSA for 1 hour at RT. The cells were labelled with V5 Tag Mouse Monoclonal Antibody (Invitrogen) at a dilution of 1:400 in 1% BSA and incubated for 3 hours at RT and then labelled with Rabbit Anti-mouse FITC Secondary Antibody (Sigma) at a dilution of 1:100 for 30 minutes at RT. Percentage of fluorescent cells was detected using a Nikon microscope at 20X magnification. 48 hours after transfection puromycin (0.5µg/ml) was added and medium containing drug was replaced on day 3 and 5. On day 8, cells were washed with PBS and fed with fresh medium without drug. Medium was replaced every

72–96 h for the remainder of the experiment. Cells were monitored daily by light microscopy for small colonies of proliferating cells. Colonies were counted 3–4 weeks after the removal of drug by staining with Coomassie Brilliant Blue. Cell clusters of 50 cells were defined as colonies.

**Angiogenesis assays**

To assess the role of angiotensin receptor signalling in the induction of tumour associated angiogenesis by melanoma cells, human microvascular endothelial cells (hCMEC/D3) were exposed to conditioned media (CM) collected from PMWK cells treated with angiotensin II alone or in combination with losartan and PD123319. To prepare conditioned medium (CM) PMWK cells were seeded onto 6 well plates (Corning, USA) at $1.5x10^5$ cells per well and allowed to adhere for 24h. Cells were washed twice with warm PBS and media replaced with 2mL per well of EBM-2 basal medium without any supplements. The EBM-2 basal medium was conditioned for 24h, then carefully collected and centrifuged at 300 RCF for 5 minutes at RT to pellet cells and debris. The CM was moved to a fresh tube and used immediately in the endothelial cell tube formation assay of angiogenesis. To perform this assay, µ-slide angiogenesis slides (Ibidi, Germany) were coated with 10µL growth factor reduced matrigel (10 mg/mL, Corning, USA) per well using cooled pipette tips to prevent early polymerisation of the matrigel. Coated slides were then placed in 10cm dishes to prevent scratching and incubated at 37°C for 30 min. During the incubation, hCMEC/D3 cells were washed twice with PBS, trypsinised and counted. hCMEC/D3 cells were aliquoted into sterile 1.5 mL micro-centrifuge tubes (Eppendorf, Germany) at $4x10^4$ cells and pelleted by 5 min centrifugation at 300 RCF. Cell pellets were re-suspended in 200µL CM and plated onto matrigel coated angiogenesis slides in triplicate (10000 cells in 50µL per well). This was done to ensure maximum exposure time of endothelial cells to the CM. Tube formation was monitored throughout the experiment and imaged at 6 hours post-plating using a TMS inverted phase contrast microscope (Nikon, Japan) with a DinoEye AM7023 eyepiece camera (Dino-Lite, Taiwan) at 2X magnification. Tube formation was analysed using the ImageJ angiogenesis analyser plugin (22).

**Zebrafish assays**

SKMEL23 cells were transplanted into 2-day-old casper zebrafish, Cells were stained prior to injection with cell tracker (CMTPX-Red, Molecular Probes) for 30min at 37°C and washed twice with PBS.

Later the cells were trypsinised and re-suspended in 500µl RPMI medium. The cells were incubated for 20 minutes on ice and 100 cells were injected into the yolk sac of 20-30 casper zebrafish/treatment (35). Following recovery for one hour at 28°C, zebrafish were maintained at 35°C, and screened for Red fluorescence at the injection site. At 48h post-injection the zebrafish were treated for 10 days with 30µM PD or EMA401 in E3 medium (1mM NaCl, 0.17mM KCl, 0.33 mM $CaCl_2$, 0.33mM $MgSO_4$, $10^{-5}$ % Methylene Blue). Tumour growth was assessed by fluorescent imaging every 48 hours.

**Clinical material**

The study was approved by the Local Research Ethical Committee, East London and City Health Authority and the Tayside Tissue Bank, under delegated authority from the Tayside Local Research Ethics Committee. Tissue for culture of CNS melanoma metastasis was obtained from Imperial College Tissue bank. Tissues and sera were used after informed consent had been obtained. In all cases, micro-dissection of tissue sections was performed to enrich for melanoma cells prior to isolation of nucleic acids. Benign pigmented nevi (n=8) from sun-exposed skin were used as control tissues. We used proteinase K digestion to isolate genomic DNA from all tissue sections. Sera were collected from patients (n=63) at the time of diagnosis of metastatic disease or during routine follow up. Sera were harvested using a standard operating procedure in which peripheral blood was subject to centrifugation immediately after venesection, then snap frozen in aliquots that were stored at -80$^{o}$ C. 200 µl units of serum were thawed immediately prior to use and genomic DNA isolated using the QIAamp DNA Blood Mini kit (Qiagen, Germany) and DNA modified with sodium bisulphite as described below.

**Methylation analysis**

The 5´ end of *AGTR1* contains a CpG island (http://www.ebi.ac.uk/Tools/emboss/cpgplot/index.html). Genomic DNA (1µg) was extracted and modified by sodium bisulphite as described previously (27, 28). Pyrosequencing was performed using the PyroMark ID System (Biotage) and primers were designed to cover a region including 7 CpG dinucleotides of the CpG island. The primers were as follows: 5'- biotin-GTTAGGATTTTAGGTAGTAG -3' (forward primer) and 5'- CTCCAACCACTCCCCAT -3' (reverse primer), amplifying a 100bp region. PCR was performed in a final volume of 30µl,

containing 0.5µM of each primer, 200µmol/L of each dNTP, 0.05 units of AmpliTaq Gold 360 DNA polymerase in buffer containing 1.5mmol/L MgCl2, 3µl of 360GC enhancer (Applied biosystems) and 2µl of bisulphite-treated DNA as template. The initial denaturation step (95°C, 10 min) was followed by 35 cycles of 30 s at 95°C, 30 s at 53°C, 40 s at 72°C, and a final extension step at 72°C for 10 min. Three µl of the PCR products were visualised by gel electrophoresis and 25µl were subjected to pyrosequencing using the reverse as sequencing primer at a final concentration of 0.5µM. Purification and subsequent processing of the biotinylated single-strand DNA was done according to the manufacturer's instructions using the Pyro Gold reagents kit (Biotage). Resulting data were analyzed and quantified with the PyroMark CpG Software (Biotage). Positive (commercial methylated DNA) and negative (placenta DNA) controls were included and treated as well as samples. Pyrograms of the control DNA were analysed to confirm complete bisulfite conversion. Sera were deemed positive if the % methylation was ≥ 5 at 3 or more Methylation Variable Positions (MVP) in the amplified fragment of the CpG island.

**Gene expression analysis**

Total RNA was extracted using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions including the optional on-column DNase step. Total RNA was reverse transcribed using random primers (Invitrogen) following the M-MLV Reverse Transcriptase protocol (Promega) and cDNA was stored at -20°C until used. TaqMan® probe-based gene expression analysis (Applied Biosystems) was used to detect *AGTR1* (assay ID: Hs00258938_m1) and *AGTR2* (assay ID: Hs02621316_s1). qPCR reactions were carried out in triplicate following the manufacturers protocol on a C1000 Thermal Cycler combined with a CFX96 detection module (Bio-Rad). 50ng of template cDNA was used per reaction. No-template controls and no-RT controls were included in each run to exclude false positives due to impurities. Expression data was normalised to the mean Ct value of the reference genes TATA-binding protein (*TBP*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and displayed as $2^{-\Delta Ct}$.

**Statistics**

Statistical analyses were performed using Prism 5 (GraphPad software, Inc., La Jolla, CA, USA). The unpaired Mann-Whitney *t*-test was used to compare methylation levels between different groups for

both cell lines and tissue samples. Significant differences in proliferation were also established by unpaired t-test.

**Conflict of interest**

The authors declare no conflict of interest.

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## Figure Legends

### Figure 1. Expression of *AGTR1* is silenced by CpG island methylation in melanoma.

A: qPCR analysis of *AGTR1* and *AGTR2* in melanoma cell lines. Data shown are mean expression +/- 1SD from 2 experiments. B: Representative pyrograms of *AGTR1* CpG island analysis in melanoma cell lines. Cell lines were subjected to pyrosequencing analysis as described in Methods. C: Summary of quantitative methylation analysis of *AGTR1* CpG island. The number denotes each individual CpG dinucleotide in the amplified fragment analysed by pyrosequencing. Five levels of methylation are represented, the level of methylation increasing with increasing intensity of shading in the circles as indicated. D: Demethylation reactivates expression of *AGTR1* in melanoma cell lines with transcriptional silencing. The indicated melanoma cell lines were grown in the presence of Trichostatin (T), Azacytidine (A) or both drugs (A & T). Expression of *AGTR1* was determined by qPCR. Data shown are mean expression relative to untreated controls (C) +/- 1SD.

**Figure 2. Methylation associated silencing of *AGTR1* is associated with metastasis in melanoma**.

A: qPCR analysis of *AGTR1* in paired primary : metastatic melanomas. The figure shows relative expression in 9 paired cases. Expression is down regulated in 7 of the 9 cases. In 3 cases expression was undetectable in the metastasis. For clarity of the figure these cases are shown as 0.01 relative expression. B: Representative pyrograms of *AGTR1* CpG island analysis in clinical cases of melanoma showing unmtheylated and methylated cases as indicated. C: *AGTR1* CpG island methylation is increased in primary melanomas which metastasize and in metastatic lesions. Genomic DNA was isolated from benign nevi (N), primary melanomas (P1-P10) which were all of BT < 1mm and did not subsequently metastasise and from high-risk primary melanomas P11-16 and their matched, subsequent metastatic lesions (M11-M16). In all cases, pyrosequencing was performed as described in Methods. The degree of methylation at each analysed MVP is represented by the intensity of shading in the circles as shown in the figure. D: Methylated genomic DNA from the *AGTR1* CpG island is detectable in the serum of patients with metastatic melanoma. The figure shows representative methylation profiles from 6 patients: 2 without metastatic disease (no metastases) and 4 with metastatic disease. The sites of metastases are: bone (Bo), lung (Lu), lymph node (LN) and sub-cutaneous (SC). The profiles show % CG methylation at each of 7 MVPs in the *AGTR1* CpG island determined by pyrosequencing. E: Expression of *AGTR1* and *AGTR2* in early passage CNS metastatic melanoma cell lines is consistent with a tumour suppressor function for *AGTR1* and oncogenic function for *AGTR2*. Novel CNS metastatic melanoma cell lines were derived as described in Methods and expression of *AGTR1* and *AGTR2* was assessed by qPCR.

**Figure 3. Antagonism of $AT_1R$ promotes melanoma proliferation**

The selective $AT_1R$ antagonist losartan promotes serum-free proliferation of the *AGTR1*-expressing melanoma cell line PMWK. PMWK cells were grown in serum-free medium without (control) or in the presence of 100μM losartan and photographed after 96 hours. B: Growth promotion of PMWK and SKMEL147 cells by losartan occurs in serum-free medium. Cells were grown in medium containing 10% FBS or in serum-free conditions in the presence (black squares) or absence (empty circles) of 100μM losartan as indicated. Cell numbers were estimated at various time points as described in Methods. Data shown are $A_{490}$ values +/- 1SD. C: Losartan-dependent proliferation in serum-free conditions requires expression of *AGTR1*. PMWK and SKMEL224 cells were grown in

serum-free medium in the presence (black squares) or absence (clear circles) of 100μM losartan as indicated and cell numbers estimated as above. Data shown are $A_{490}$ values +/- 1SD.

**Figure 4. *AGTR1* is a growth suppressor in melanoma but AngiotensinII promotes melanoma proliferation.**

A: Stable knock down of *AGTR1* promotes low serum growth of PMWK cells. PMWK cells stably expressing the *AGTR1* shRNA vector or non-silencing control vector were cultured in 10% FBS in the absence (-) or presence (+) of doxycycline (2μg/ml) and knockdown of *AGTR1* was assessed by qPCR after 48 hours (left hand figure) and western blotting (middle figure). Image analysis (right hand figure) confirms knock down. Cells stably expressing either the control plasmid (control) or AGTR1 knockdown plasmid (AGTR1) were then grown in 10% FBS, 2% FBS or 1% FBS in the absence (-) or presence (+) of doxycycline as shown and $A_{490}$ determined after 72 hours. Data shown are mean $A_{490}$ values +/- 1SD. C: Ectopic expression of *AGTR1* inhibits proliferation in melanoma cells lacking endogenous expression. SKMEL224, SKMEL23 and PMWK cells were transfected with 0, 100 or 1000 ng of pcDNA3AGTR1 then grown in medium with 10% serum together with G418. Data shown are colony number +/- 1SD relative to cells receiving vector only from at least two experiments. D: Dose : response effect of AngII on proliferation of melanoma cell lines. The upper panel shows growth in 10% foetal bovine serum in which exogenous AngII has no effect on proliferation. The lower panel shows growth in 0% serum in which the proliferation of PMWK and SKMEL23, but not that of SKMEL224, is promoted in a dose-dependent manner by AngII. Cell lines were grown in the presence of the indicated concentrations of AngII and proliferation determined as described in Methods. Data shown are mean $A_{490}$ values +/- 1SD. E: Y6AII, a potent and highly specific agonist of $AT_2R$, promotes serum-free proliferation of cells expressing *AGTR2*. PMWK cells were grown in serum-free medium in the presence of increasing concentrations of Y6AII or 100 pM AngII as indicated and proliferation assessed. UC1: at start of experiment; UC5: untreated control after 5 days. Data shown are A490 values +/- 1SD.

**Figure 5. Inhibition of AT2R suppresses proliferation of melanoma cell lines**

A: Effect of PD123319 on proliferation of melanoma cell lines. PMWK (AGTR1 + / AGTR2 +), SKMEL23 (AGTR1 - / AGTR2 +) and SKMEL 224 (AGTR1 - / AGTR2 -) cells were grown in

either 10% serum (left hand panels) or 0% serum (right hand panels). Cells grown in 0% serum were either untreated control cells (clear circles), treated with AngII (A, black squares) or AngII + PD123319 (A + PD123319, black diamonds). B: Suppression of proliferation of AGTR2 expressing melanoma cells by inhibition of $AT_2R$. SKMEL23 cells were grown in medium containing1% serum in the absence of exogenous AngII but in the presence of varying concentrations of either PD123319 or EMA401 as shown and cell numbers estimated by measurement of A490. C: $AT_2R$ blockade inhibits melanoma growth in vivo. SKMEL23 cells were injected into Zebrafish yolksac as described in Methods. -: control fish not injected; +U injected but untreated control; +EMA injected and grown in the presence of 30μM EMA.

**Figure 6. Antagonism of $AT_2R$ inhibits proliferation and angiogenesis and potentiates targeted therapy in melanoma.**

A: Blocking AT2R inhibits angiogenesis. Angiogenesis assays in PMWK and SKMEL23 cells were performed as described in Methods. The upper panels show representative appearances of SKMEL23 untreated controls (UC) and cells exposed to EMA401 and the lower panels PMWK untreated controls (UC) and cells exposed to PD123319. B: Quantitative analysis of total segment length (upper panels) and number of meshes (lower panels). PMWK and SKMEL23 cells were treated in the indicated way (CM: conditioned medium; AngII: 100 pM angiotensinII; LOS: 100μM losartan; PD30: 30μM PD123319; Y6: 100μM Y6AII; EMA30: 30μM EMA401). Data are mean +/- 1SD. C: Effect of AT2R antagonism on sensitivity to BRAF inhibition in melanoma. SKMEL23 (*BRAF* wild-type), A375, WM164 and WM793 (all *BRAF* V600 mutation positive) cells were grown in 1% serum in the presence of EMA401 (30μM), dabrafenib (5nM) or both agents as shown and cell numbers directly counted. Data shown are mean relative cell number +/- 1SD from 2 independent experiments.

Table 1:

| Cell line | Description | *BRAF* / *NRAS* | *AGTR1* | U or M |
|---|---|---|---|---|
| HEMA | Normal human melanocytes | Wt / Wt | 3 | U |
| HEMN1 | Normal human melanocytes | Wt / Wt | 1 | U |
| SBCL2 | RGP | Wt / Q61L | 6 | U |
| PMWK | RGP | Wt / Wt | 2 | U |
| WM35 | RGP | V600E/ Wt | 15 | U |
| WM-902.6 | VGP | V600E/ Wt | 1 | U |
| SKMel224 | VGP | Wt / Q61R | 35 | M |
| SKMel505 | VGP | Wt / Wt | 94 | M |
| WM-266-4 | Metastatic melanoma | V600D / Wt | 9 | U |
| SKMel2 | Metastatic melanoma | Wt / Q61R | 22 | M |
| SKMel23 | Metastatic melanoma | G466A / Wt | 94 | M |
| SKMel30 | Metastatic melanoma | Wt / Q61R | 90 | M |
| SKMel147 | Metastatic melanoma | Wt / Q61R | 3 | U |
| SKMel173 | Metastatic melanoma | Wt / Q61K | 41 | M |
| SKMel501 | Metastatic melanoma | V600E / Wt | 2 | U |
| COLO-829 | Metastatic melanoma | V600E / Wt | 19 | U |
| C8161 | Metastatic melanoma | Wt / Wt | 24 | M |
| WM239A | Metastatic melanoma | V600D/Wt | 7 | M |
| A375M | Metastatic melanoma | V600E/Wt | 3 | U |

Abbreviations: RGP: Radial Growth Phase; SSM: Superficial Spreading Melanoma; VGP: Vertical Growth Phase Melanoma; Wt: Wild Type. M: methylated; U: unmethylated.

FIGURE 1

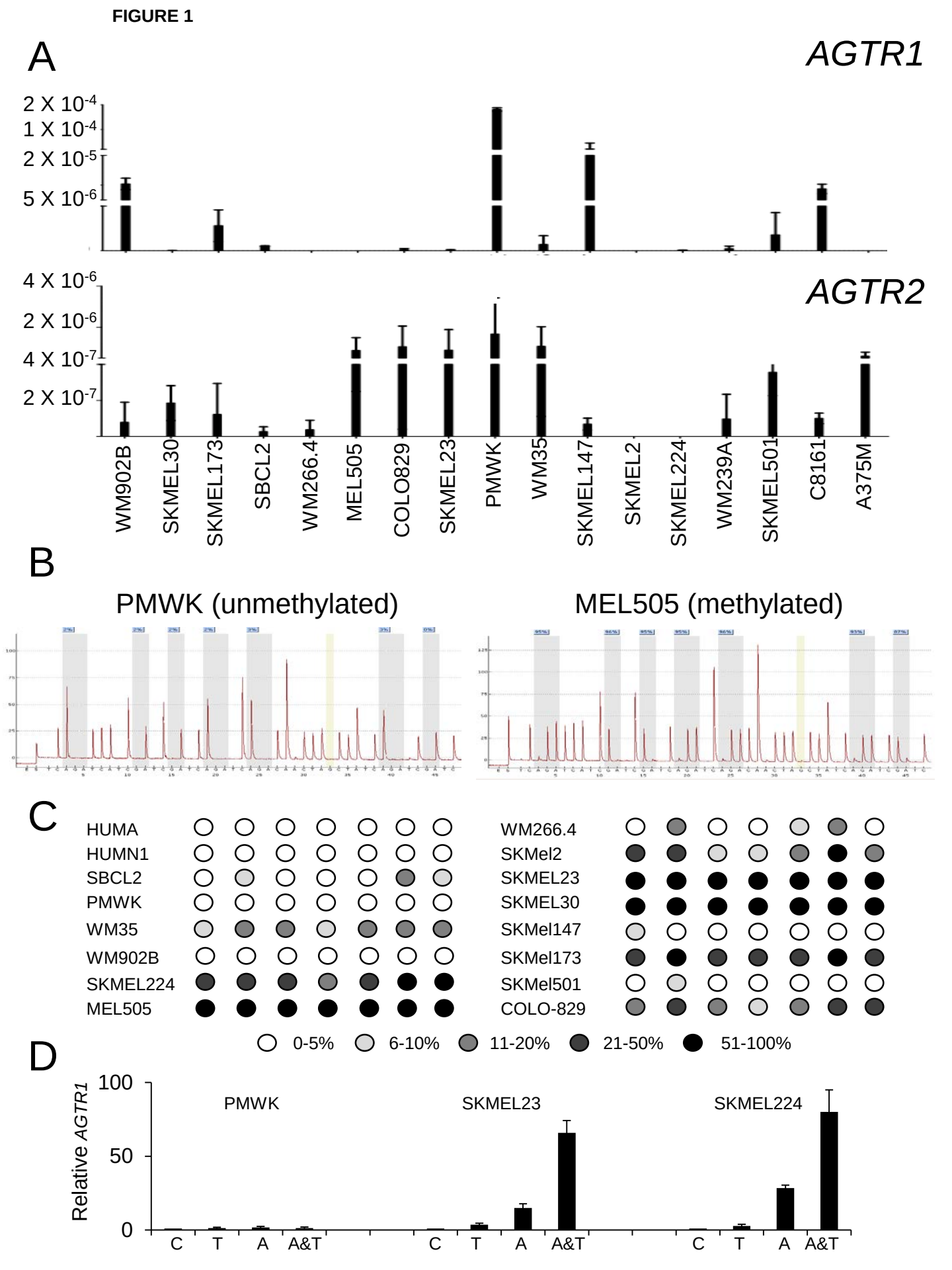

A
AGTR1
2 X 10-4
1 X 10-4
2 X 10-5
5 X 10-6
AGTR2
4 X 10-6
2 X 10-6
4 X 10-7
2 X 10-7
WM902B
SKMEL30
SKMEL173
SBCL2
WM266.4
MEL505
COLO829
SKMEL23
PMWK
WM35
SKMEL147
SKMEL2
SKMEL224
WM239A
SKMEL501
C8161
A375M
B
PMWK (unmethylated)
MEL505 (methylated)
C
HUMA
HUMN1
SBCL2
PMWK
WM35
WM902B
SKMEL224
MEL505
WM266.4
SKMel2
SKMEL23
SKMEL30
SKMel147
SKMel173
SKMel501
COLO-829
0-5%
6-10%
11-20%
21-50%
51-100%
D
Relative AGTR1
100
50
0
PMWK
SKMEL23
SKMEL224
C
T
A
A&T

FIGURE 2

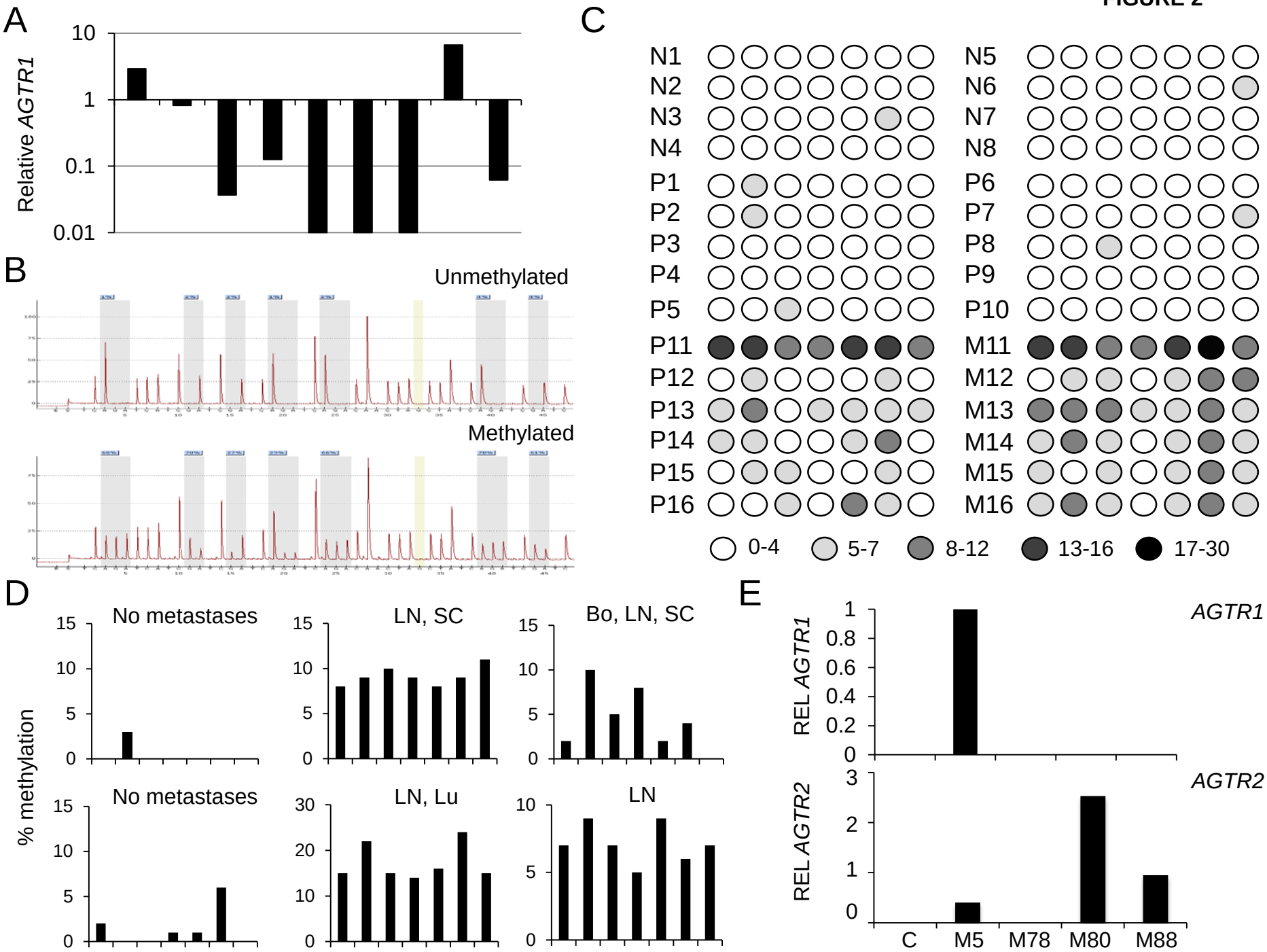

A
Relative AGTR1
10
1
0.1
0.01
B
Unmethylated
Methylated
C
N1
N2
N3
N4
P1
P2
P3
P4
P5
P11
P12
P13
P14
P15
P16
N5
N6
N7
N8
P6
P7
P8
P9
P10
M11
M12
M13
M14
M15
M16
0-4
5-7
8-12
13-16
17-30
D
% methylation
No metastases
LN, SC
Bo, LN, SC
No metastases
LN, Lu
LN
E
REL AGTR1
AGTR1
REL AGTR2
AGTR2
C
M5
M78
M80
M88

**FIGURE 4**

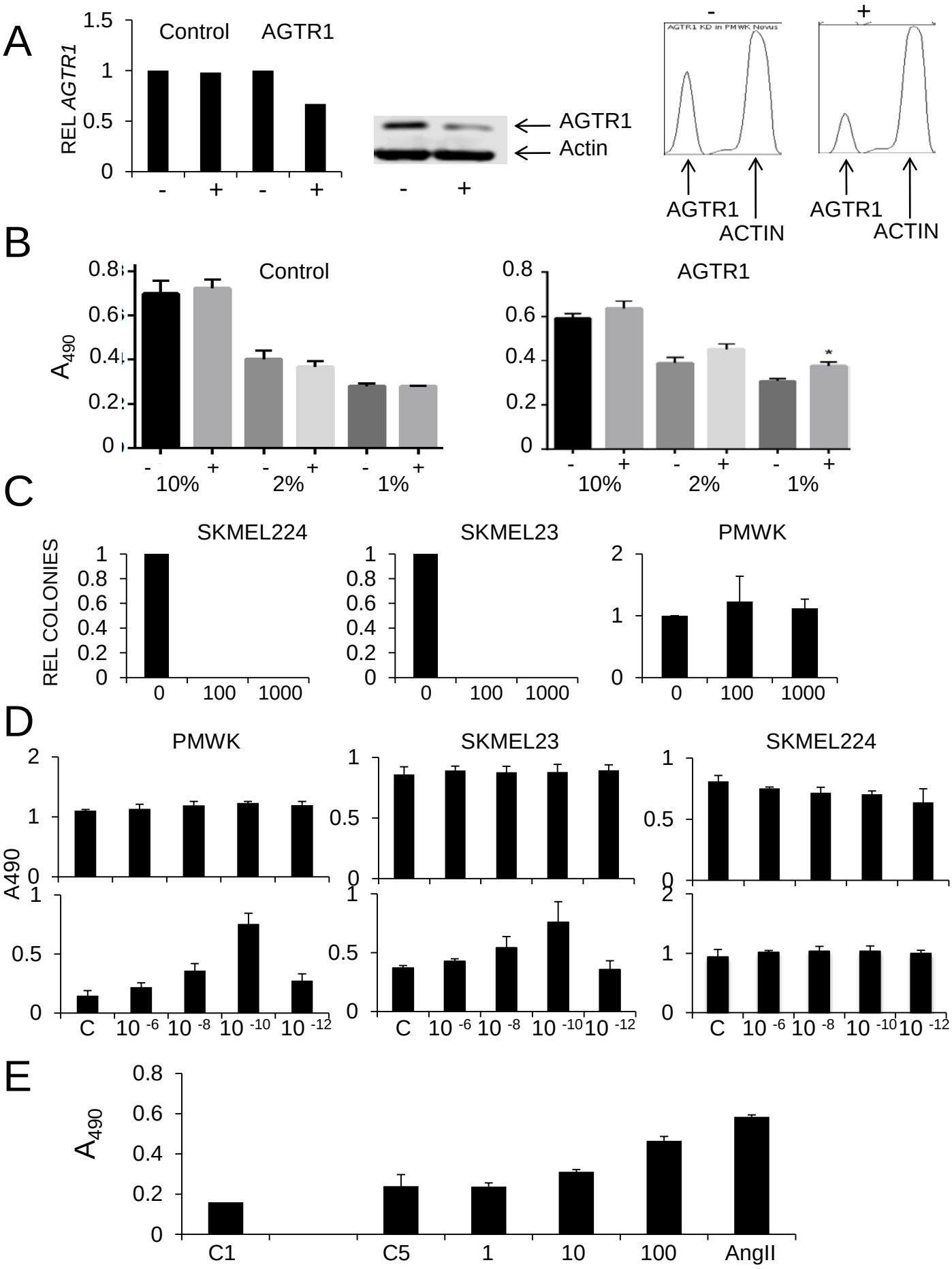
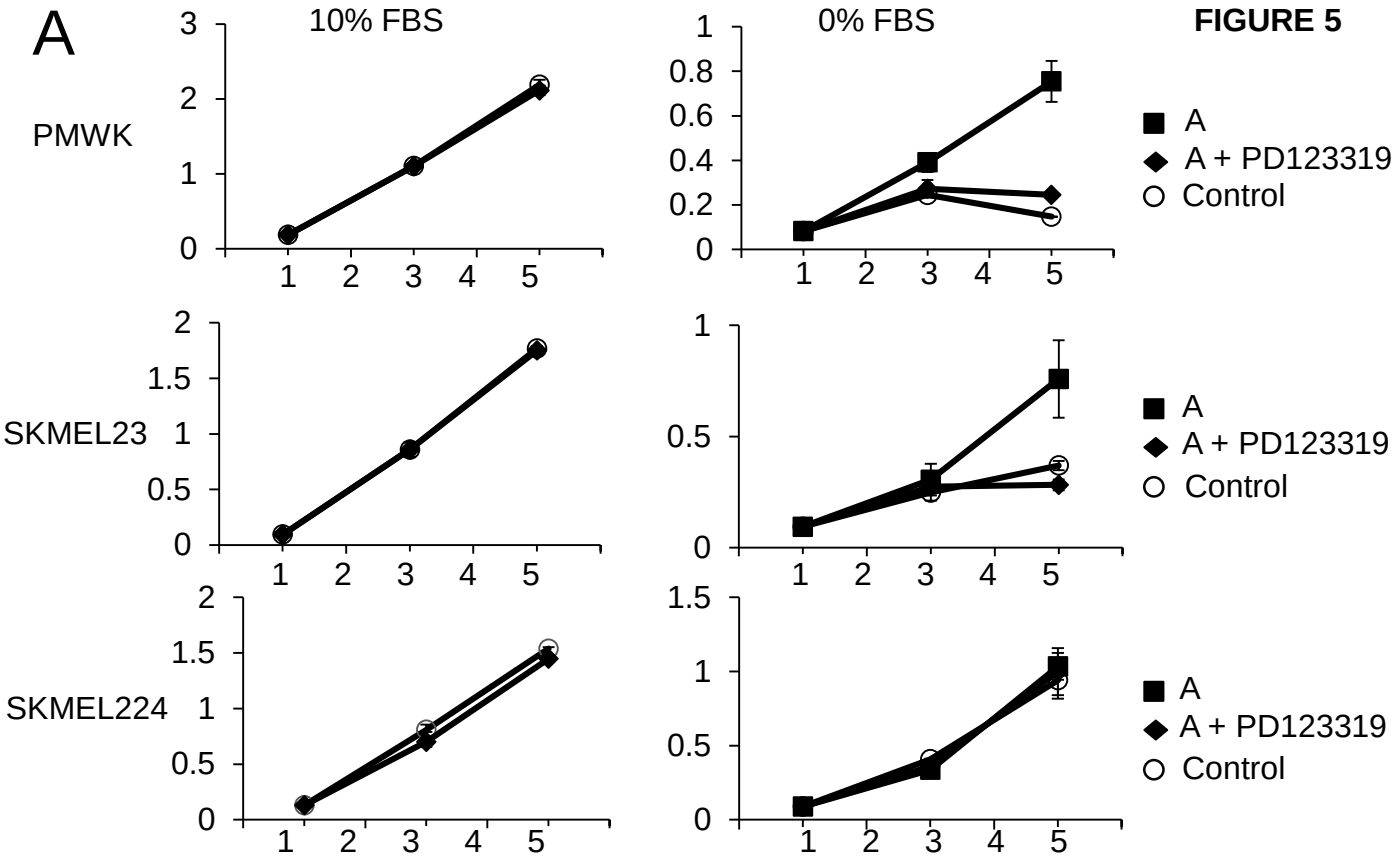
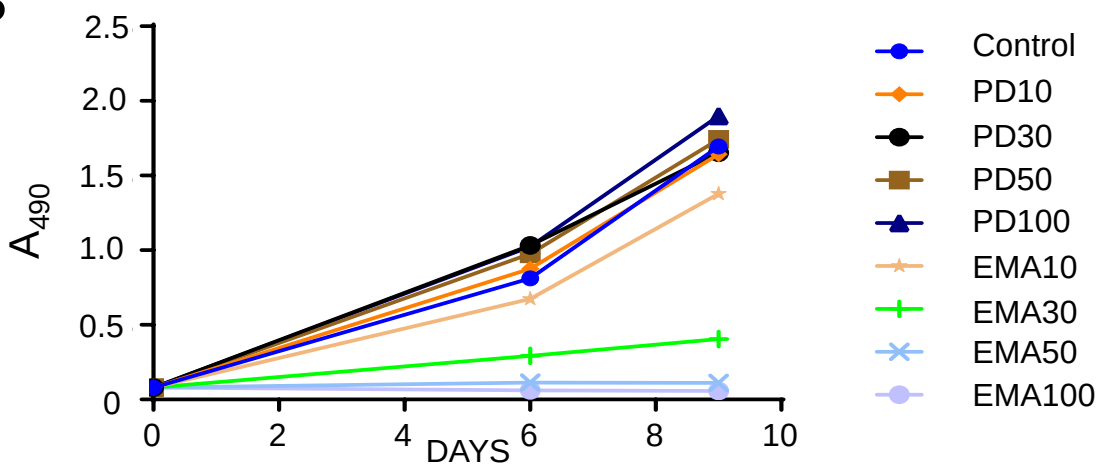

A
Control
AGTR1
REL AGTR1
1.5
1
0.5
0
- + - +
AGTR1
Actin
- +
AGTR1 KD in PMWK Novus
-
+
AGTR1
ACTIN
AGTR1
ACTIN
B
Control
AGTR1
A490
0.8
0.6
0.4
0.2
0
- + - + - +
10% 2% 1%
*
C
SKMEL224
SKMEL23
PMWK
REL COLONIES
0 100 1000
D
PMWK
SKMEL23
SKMEL224
A490
C 10 -6 10 -8 10 -10 10 -12
E
A490
C1
C5
1
10
100
AngII

FIGURE 5

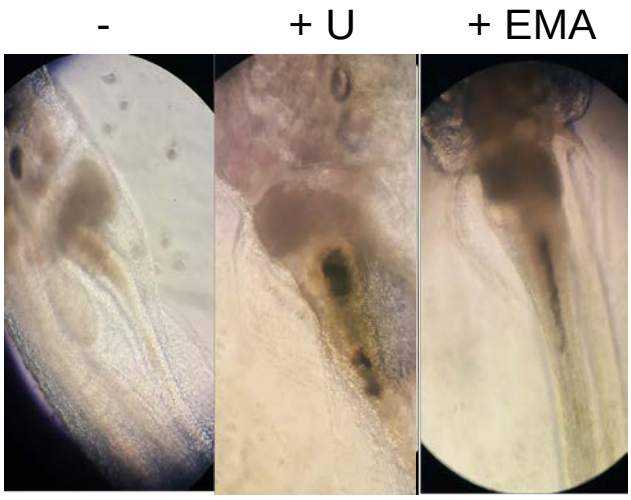
A


10% FBS
0% FBS
PMWK
SKMEL23
SKMEL224
A
A + PD123319
Control

B


$A_{490}$
DAYS
Control
PD10
PD30
PD50
PD100
EMA10
EMA30
EMA50
EMA100

C


-
+ U
+ EMA

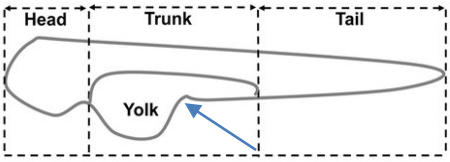

Head
Trunk
Tail
Yolk

**FIGURE 3**

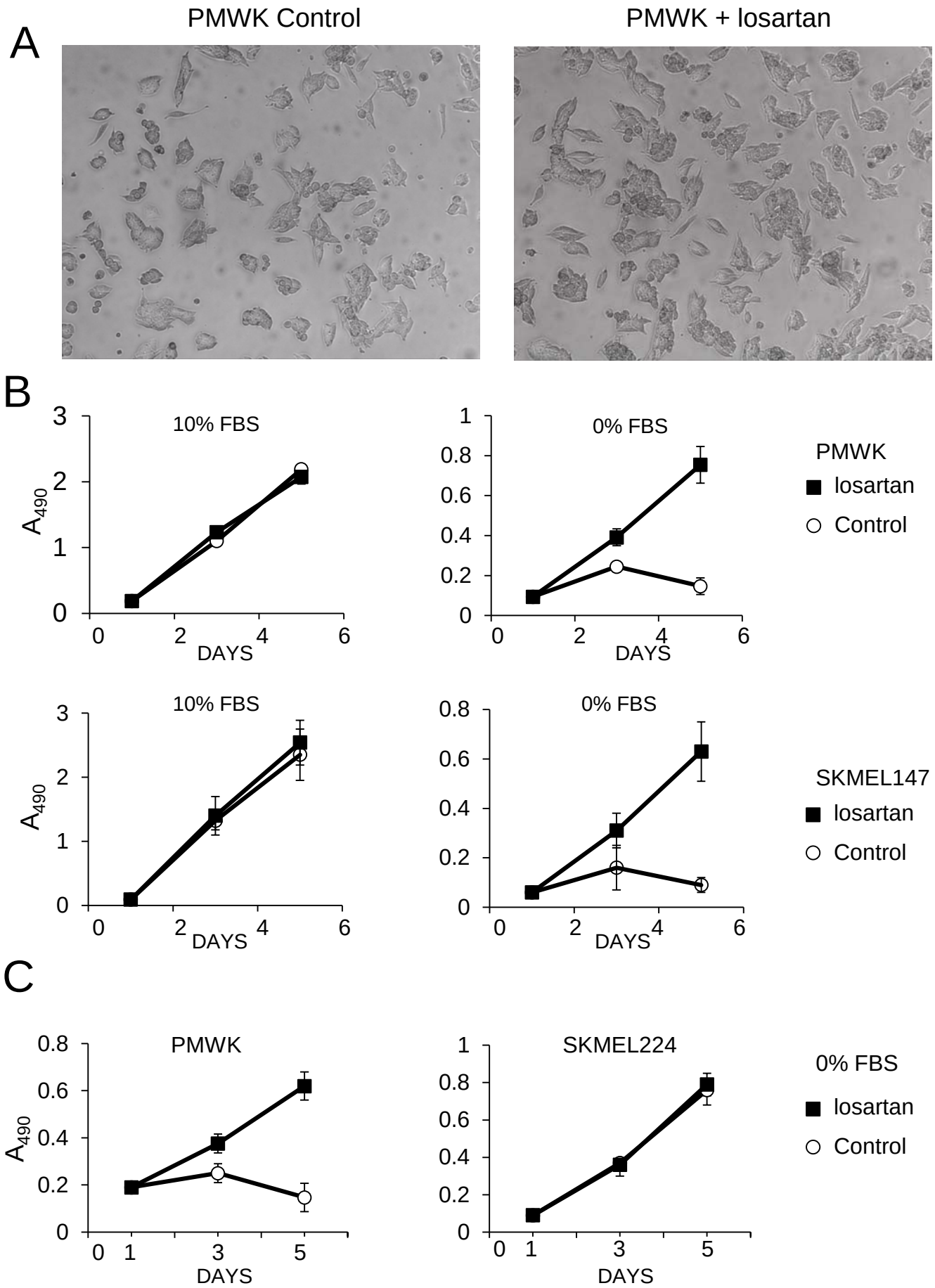
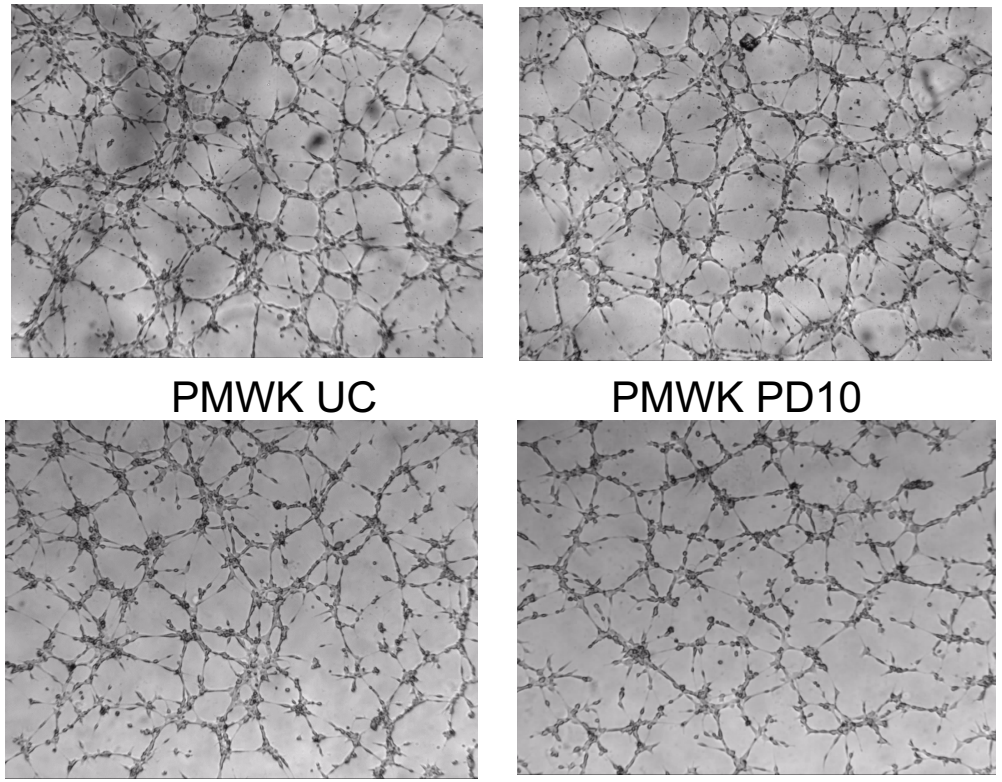
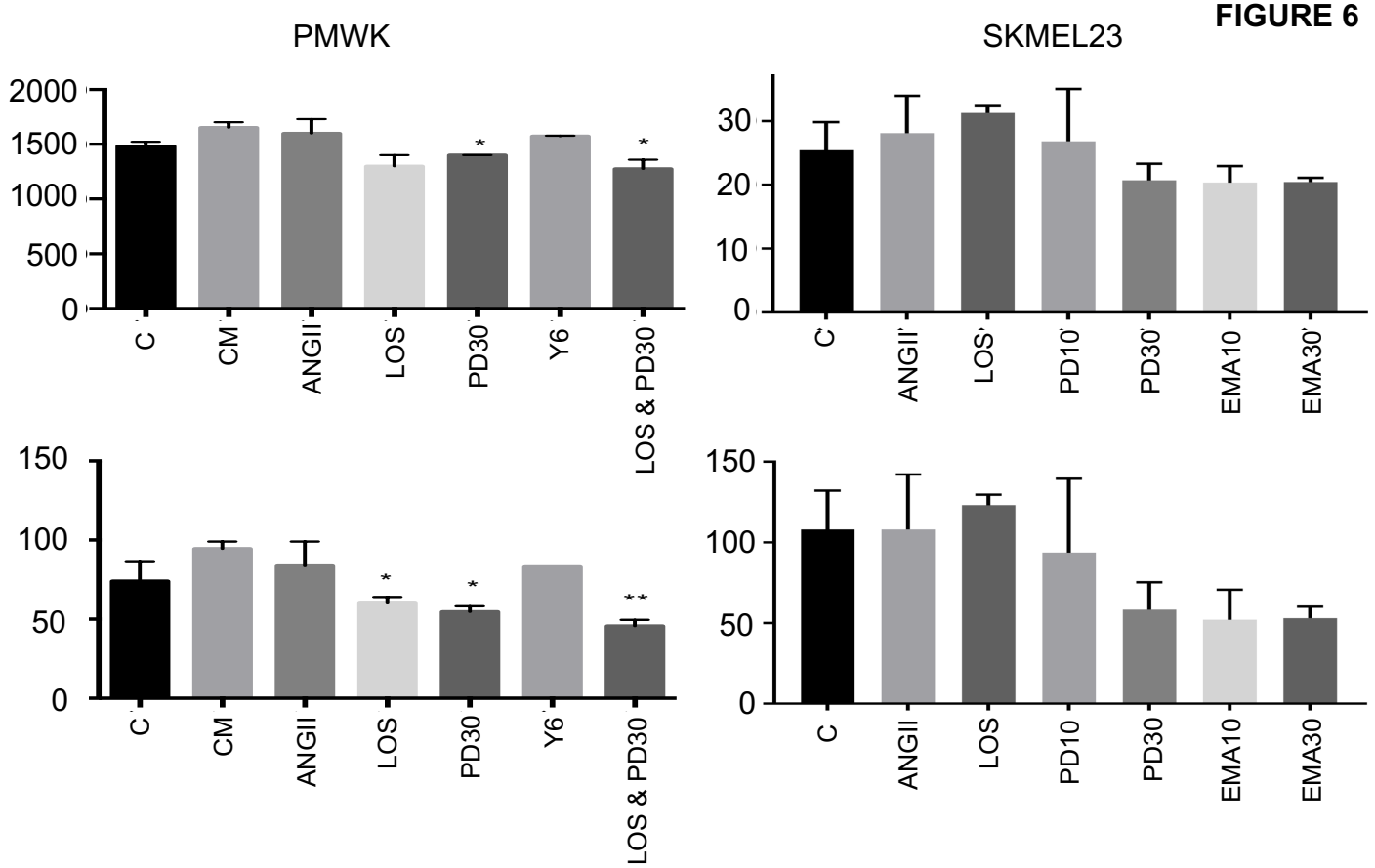

A
PMWK Control
PMWK + losartan
B
10% FBS
0% FBS
$A_{490}$
DAYS
PMWK
losartan
Control
SKMEL147
C
PMWK
SKMEL224
0% FBS

FIGURE 6

A


SKMEL23 UC
SKMEL23 EMA30
PMWK UC
PMWK PD10

B


PMWK
SKMEL23
2000
1500
1000
500
0
30
20
10
150
100
50
C
CM
ANGII
LOS
PD30
Y6
LOS & PD30
PD10
EMA10
EMA30
*
**

C

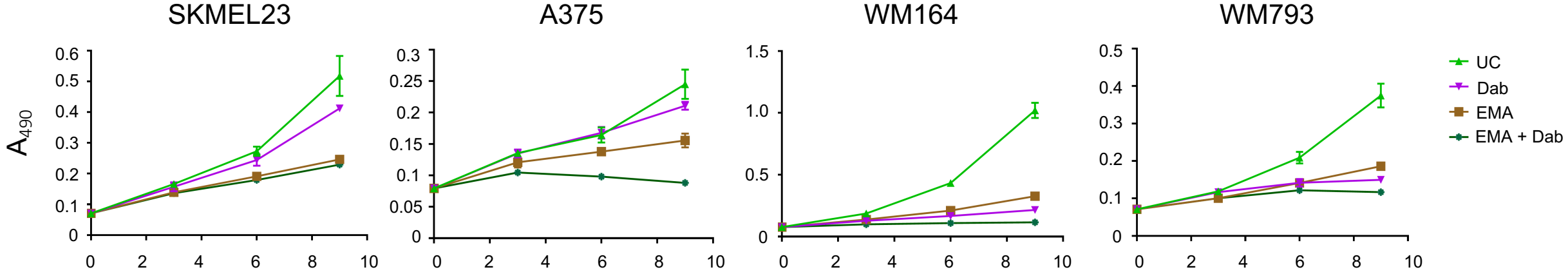

SKMEL23
A375
WM164
WM793
$A_{490}$
UC
Dab
EMA
EMA + Dab

Table 1

| Cell line | Description | *BRAF* / *NRAS* | *AGRT1* % methylation | U or M |
|---|---|---|---|---|
| HEMA | Normal human melanocytes | Wt / Wt | 3 | U |
| HEMN1 | Normal human melanocytes | wt / Wt | 1 | U |
| SBCL2 | Radial growth phase melanoma | Wt / Q61L | 6 | U |
| PMWK | Radial growth phase melanoma | Wt / Wt | 2 | U |
| WM35 | Radial growth phase melanoma | V600E / Wt | 15 | U |
| WM-902.6 | Vertical growth phase melanoma | V600E / Wt | 1 | U |
| SKMel224 | Vertical growth phase melanoma | Wt / Q61R | 35 | M |
| SKMEL505 | Vertical growth phase melanoma | Wt / Wt | 94 | M |
| WM-266.4 | Metastatic melanoma | V600D / Wt | 9 | U |
| SKMEL2 | Metastatic melanoma | Wt / Q61R | 22 | M |
| SKMEL23 | Metastatic melanoma | G466A / Wt | 94 | M |
| SKMEL30 | Metastatic melanoma | Wt / Q61R | 90 | M |
| SKMEL147 | Metastatic melanoma | Wt / Q61R | 3 | U |
| SKMEL173 | Metastatic melanoma | Wt / Q61K | 41 | M |
| SKMEL501 | Metastatic melanoma | V600E / Wt | 2 | U |
| COLO-829 | Metastatic melanoma | V600E / Wt | 19 | U |
| C8161 | Metastatic melanoma | Wt / Wt | 24 | M |
| WM-239A | Metastatic melanoma | V600D / Wt | 7 | U |
| A375M | Metastatic melanoma | V600E / Wt | 3 | U |

Abbreviations Wt: wild-type; U: unmethylated; M: methylated.