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ORIGINAL PAPER

Angiotensin-(1–7) inhibits in vitro endothelial cell tube formation in human umbilical vein endothelial cells through the $AT_{1–7}$ receptor

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Abstract Angiotensin-(1–7) is increased in the circulation during human pregnancy, but its functional role is unknown. Recent studies suggested that it opposes angiotensin II mediated vascular growth. Because angiogenesis is critical to normal embryonic development during human pregnancy, this study assessed the in vitro effects of angiotensin-(1–7) on human umbilical vein endothelial cell tube formation. The blocking effects of the angiotensin-(1–7) receptor antagonist, D-[Alanine^7]-Ang-(1–7), and angiotensin II receptor AT_1 and AT_2 antagonists, losartan and PD123319, on tube formation were measured by counting tube branch points. Human umbilical vein endothelial cells were cultured in EGM-2 medium and treated with angiotensin-(1–7) (0.17 nM–17 μM) for 18 h. Angiotensin-(1–7) inhibited tube formation by 24% ($P < 0.01$) at all doses tested. Treatment with 1.7 μM angiotensin-(1–7) plus 17 μM D-[Alanine^7]-Ang-(1–7) resulted in the reversal of angiotensin-(1–7) mediated inhibition of tube formation ($P < 0.05$). Losartan (17 μM) also reversed the angiotensin-(1–7) mediated inhibition of tube formation ($P < 0.05$). Tube formation was unaffected by PD123319. These results suggest that angiotensin-(1–7) has an anti-angiogenic effect on human umbilical vein endothelial cells through a unique $AT_{1–7}$ receptor that is sensitive to losartan, indicating that angiotensin-(1–7) may play an important role in the regulation of vascular growth in the placenta during pregnancy.

Keywords AT_1 · A779 · D-Ala · Losartan · Angiotensin receptors · Renin–angiotensin system · Placenta · HUVEC · Pregnancy · Angiogenesis

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Introduction

Pregnancy is characterized by an increase in the components of the renin–angiotensin system (RAS) [1–8]. However, the physiological mechanisms of stimulated RAS activity are unknown in both normal pregnancy and in hypertensive disorders of pregnancy. The recently discovered peptide of the RAS, angiotensin-(1–7) [Ang-(1–7)] [9, 10], has been shown in previous studies to increase in the circulation in the third trimester of pregnancy [7] and in urine throughout gestation of pregnancy [8]. A balance of the two biologically active peptides of the RAS, angiotensin II (Ang II), a potent vasoconstrictor, and Ang-(1–7), a vasodilator, is essential for the maintenance of normal pregnancy. Most of the actions of Ang II, including vasoconstriction, aldosterone stimulation, angiogenesis stimulation, and cell growth, are mediated by the AT_1 receptor. Only recently the AT_2 receptor was described and is shown to be upregulated during fetal development, and implicated in the reduction in neointima formation after vascular injury, reduction in endothelial cell growth and migration, and vasodilation [11–14]. Recently, a selective Ang-(1–7) receptor antagonist [D-Alanine^7-Angiotensin-(1–7)] (A779 or D-Ala) that does not interact with either the AT_1 or the AT_2 receptor was characterized. Using this antagonist, a role for Ang-(1–7) in blood pressure regulation, vasodilation, and electrolyte excretion has been shown [15–18]. More recently, Santos

et al. [19, 20] showed that Ang-(1–7) binds with high affinity to the *Mas*-G protein coupled receptor. Previously shown Ang-(1–7) functions, including a vasodilator response and anti-diuresis in water-loaded animals, were absent in *Mas* knockout mice [19], which provides evidence that is consistent with the *Mas* receptor being the AT_{1-7} receptor. Previous studies showed that human urinary and plasma Ang-(1–7) levels increase during late pregnancy, suggesting that Ang-(1–7) may play a role in the regulation of blood pressure during pregnancy [7, 8]. In addition, we have previously shown that Ang-(1–7) and its generating enzyme, angiotensin converting enzyme 2 (ACE2), are expressed in the human placenta, specifically in the syncytiotrophoblast, cytotrophoblast, endothelium, and vascular smooth muscle [21]. Ang-(1–7) expression was increased during the first trimester of spontaneously aborted pregnancies when compared to third trimester normal pregnancies [21]. These findings indicate that an increase of Ang-(1–7) in early pregnancy, a time in which the invasion of uterine spiral arteries and the development of the placenta are crucial, might play an important role in regulating the angiogenic process during placental development. In addition, we have found that Ang II is elevated while Ang-(1–7) does not change in the third trimester chorionic villi from preeclamptic women [22]. This indicates that the balance of Ang II/Ang-(1–7) is shifted toward Ang II, the vasoconstrictor and angiogenic protein of the RAS, in preeclampsia. The balance of Ang II/Ang-(1–7) is essential for the regulation of the angiogenic process in the placenta during pregnancy. While Ang II is needed to increase blood vessel growth in the placenta, Ang-(1–7)'s anti-angiogenic effects may play a role in keeping vessel growth under control or if present in excess may prevent necessary blood vessel growth. These two peptides working together may play an integral part in the regulation of the angiogenic process, including cell migration, proliferation, and vessel formation, during normal pregnancy.

The RAS has been shown to be a modulator of blood vessel growth and angiogenesis. While Ang II, acting though the AT_1 receptor, has been shown to induce the over expression of VEGF in several cell types causing an increase in angiogenesis [23–27], Ang-(1–7) has been shown to inhibit angiogenesis. Ang-(1–7) inhibited angiogenesis in cultured rat thoracic aortic vascular smooth muscle cells through the activation of the AT_{1-7} receptor [28, 29]. Opposing actions of Ang-(1–7) (inhibitory) and Ang II (stimulatory) on angiogenesis and fibrovascular tissue growth were observed in a mouse sponge model of angiogenesis [30, 31]. The anti-angiogenic effect of Ang-(1–7) was not reversed by the AT_1 and AT_2 receptor antagonists, losartan and PD123319; however, the Ang-(1–7) selective antagonist, A779, blocked the anti-angiogenic effects [30]. Although Ang-(1–7) has previously been shown to be anti-angiogenic, the effect of Ang-(1–7) on human umbilical vein endothelial cells (HUVECs) is unknown. Therefore, the objective of this study is to determine the receptor mediated effect of Ang-(1–7) treatment on HUVEC tube formation by treating with or without specific angiotensin receptor antagonists. It is hypothesized that Ang-(1–7) will inhibit the tube formation and growth of HUVECs by a specific Ang-(1–7) receptor.

Results

The effect of vehicle or Ang-(1–7) (1.7 μM) treatment on HUVEC tube formation is illustrated in Fig. 1a. The quantification of the number of branch points seen after treatment with six doses of Ang-(1–7) (0.17 nM–17 μM) is shown in Fig. 1b. The calculated number of branch points was expressed as a percent of control (vehicle-treated HUVECs). Treatment with all six concentrations of Ang-(1–7) resulted in an inhibition in HUVEC tube formation. There was approximately a 24% decrease in endothelial cell tube growth when HUVECs were treated with increasing doses of Ang-(1–7) ($P < 0.01$).

In order to determine the angiotensin receptor mediating the inhibitory effects of Ang-(1–7) on tube growth, cells were treated with a combination of Ang-(1–7) (1.7 μM) and angiotensin receptor antagonists (17 μM) including losartan, PD123319, or A779, which are responsible for blocking the binding of the AT_1, AT_2, and AT_{1-7} receptor, respectively. Shown in Fig. 2, Ang-(1–7) (1.7 μM) alone inhibited HUVEC tube formation by about 26% as was seen in Fig. 1 ($P < 0.01$). The inhibitory effect of Ang-(1–7) on HUVEC tube growth was reversed with A779 ($P < 0.05$). In addition, we found that treatment with losartan resulted in a reversal of the Ang-(1–7) mediated inhibition in a similar way to that of A779 ($P < 0.05$). Treatment with PD123319 had no effect on the Ang-(1–7) mediated inhibition of tube growth. The three angiotensin receptor antagonists alone did not show any effect on tube formation and were not significantly different from the control vehicle-treated HUVECs.

Discussion

This study is the first to show that Ang-(1–7) causes a decrease in HUVEC tube formation via an in vitro assay that is often used as a tool to study angiogenesis. In addition, we showed that the reduction in HUVEC tube formation by Ang-(1–7) is reversed by treatment with the AT_{1-7} receptor antagonist, A779. Treatment of HUVECs with the AT_1 receptor antagonist, losartan, also reversed the Ang-(1–7) mediated decreased in tube formation.

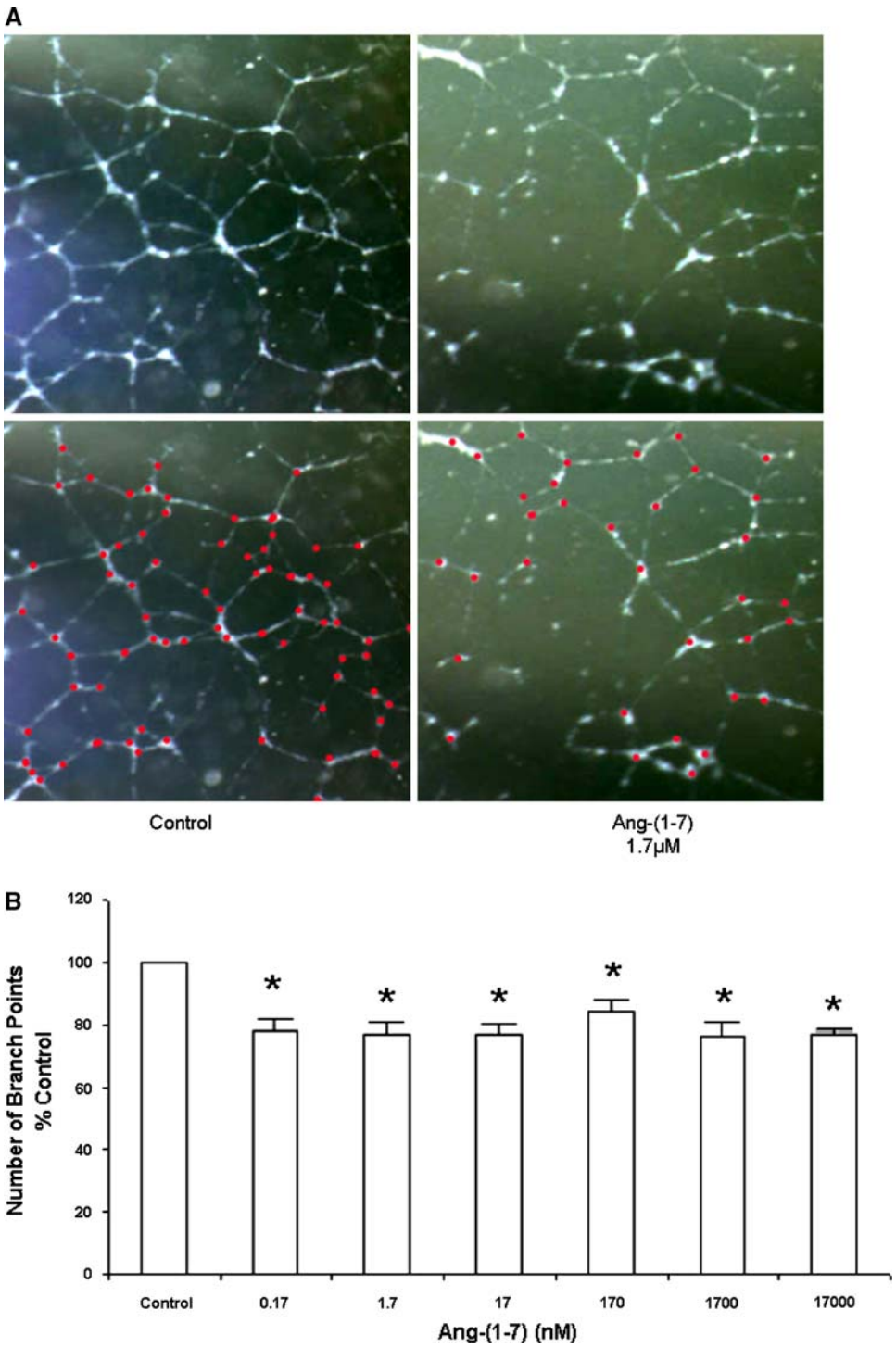

Fig. 1 (**a**) Representative micrographs of vehicle-treated control and Ang-(1–7) (1.7 μM) treated HUVECs. Bottom two micrographs illustrate the procedure used to quantify the number of HUVEC branch points (identified by the red dots) as an analysis of HUVEC tube formation. (**b**) Effect of Ang-(1–7) on HUVEC tube formation expressed as number of branch points. $n = 5$ per group, $^*P < 0.01$ vs. control (vehicle-treated HUVECs)

However, treatment with the AT_2 receptor antagonist, PD123319, had no effect on the reduction in tube formation by Ang-(1–7). These results indicate that the Ang-(1–7) mediated decrease in HUVEC tube formation occurred through an A779-site that is also a losartan-sensitive site.

The Ang-(1–7) inhibition of HUVEC tube formation is consistent with in vitro studies done in cultured rat thoracic aortic vascular smooth muscle cells [28] and in in vivo studies done in the mouse sponge model of angiogenesis [30, 31]. Treatment with Ang-(1–7) resulted in a diminished proliferative activity of the sponge induced fibrovascular tissue [31]. In addition, it was shown that Ang-(1–7) exerted its anti-angiogenic effect through the specific AT_{1-7} receptor since the AT_{1-7} receptor antagonist, A779, was able to abolish the anti-angiogenic effect of Ang-(1–7). In that study, it was determined that the

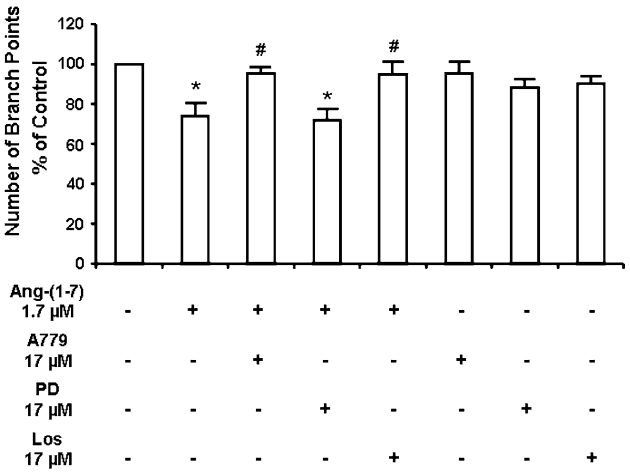

Fig. 2 The effects of Ang-(1–7) (1.7 μM) or vehicle with or without angiotensin receptor antagonists A779 (17 μM), PD123319 (17 μM), or losartan (17 μM) on HUVEC tube formation expressed as number of branch points. $n = 7–10$ per group, $^*P < 0.01$ versus control (vehicle-treated HUVECs), $\#P < 0.05$ versus Ang-(1–7) (1 μM) treatment. PD, PD123319; Los, losartan

anti-angiogenic effect of Ang-(1–7) was significantly blocked by treatment with nitric oxide (NO) synthase inhibitors (aminoguanidine and L-NAME), indicating that Ang-(1–7) has an inhibitory effect through inducible and constitutive NO release [30]. Sampaio et al. [32] showed that human aortic endothelial cells express the $AT_{1–7}$ receptor, *Mas* receptor, which in response to Ang-(1–7) stimulates the phosphorylation of eNOS and is blocked by A779. Ang-(1–7) may be inhibiting endothelial cell tube formation in HUVECs through the same potential mechanisms, by increasing the release of NO. In the present study, we did not see a concentration-dependent response in the Ang-(1–7) mediated inhibition of HUVEC tube growth. Although several studies have reported concentration-dependent responses to Ang-(1–7) [33, 34], our analysis of their data suggests that the concentration-dependent effect is somewhat questionable since the values at the different concentrations were not statistically different from each other. The lack of a concentration-dependent response in our study indicates that Ang-(1–7) has modest effects on HUVEC growth. However, the presence of Ang-(1–7) in the placenta during pregnancy indicates that it might play an important role in restricting vessel growth.

Our study also investigated the subtype of angiotensin receptors responsible for the Ang-(1–7) mediated inhibition of HUVEC tube formation. Treatment with the $AT_{1–7}$ selective receptor antagonist, A779, resulted in a significant reversal of the Ang-(1–7)-induced inhibition of tube growth. These results indicate that Ang-(1–7) is acting predominantly through its specific $AT_{1–7}$ receptor. This agrees with previous studies that have shown that Ang-(1–7) acts in the circulation and endothelium at the $AT_{1–7}$ receptor [35–38]. Further support as evidence of the specificity of the binding of A779 to the $AT_{1–7}$ receptor in endothelial cells was shown in binding studies done in bovine aortic endothelial cells (BAECs). The ^{125}I-Ang-(1–7) binding site on BAECs was competed for by A779 [39].

In addition to treatment with the $AT_{1–7}$ receptor antagonist, A779, treatment with the AT_1 receptor antagonist, losartan, also abolished the decrease in tube formation seen with Ang-(1–7) treatment. This result has not been seen in previous studies investigating the effects of Ang-(1–7) on cell growth. However, studies investigating the actions of Ang-(1–7) in other regions or tissue types have previously described that Ang-(1–7)'s effects can be blocked by a losartan-sensitive site in addition to A779 blockade [40–42]. A previous study done in our laboratory found similar results to this study in that Ang-(1–7) caused dilation of rat mesenteric arteries that was blocked by administration of either A779 or losartan [43]. Studies showing a reversal of Ang-(1–7)'s effect by A779 and losartan have suggested that high doses of Ang-(1–7) may be acting on AT_1 receptors [44]. Additional studies conducted in our lab testing lower concentrations of Ang-(1–7) (1.7 nM) and losartan (17 nM) also reversed the Ang-(1–7) mediated inhibition ($101 \pm 5\%$ of control with losartan vs. 100% vehicle treated, $n = 9$) of endothelial cell tube formation. These results provide support that this effect is not due to Ang-(1–7) acting at the AT_1 receptor. In addition, it has been previously shown that Ang-(1–7) has a poor affinity for the AT_1 receptor [39, 45]. The affinity of Ang-(1–7) to the AT_1 receptor is approximately 15-fold less than that of Ang II [45]. In addition, Ang II has been shown in numerous studies to increase or induce angiogenesis through the AT_1 receptor [46, 47]. Mice lacking the AT_1 receptor showed a reduction in angiogenesis induced by hind limb ischemia [48] or myocardial infarction [49] when compared to wild type mice. Thus, it would seem unlikely for Ang-(1–7) to be exerting its anti-angiogenic effects through the AT_1 receptor. Therefore, our data suggest that Ang-(1–7) is causing an inhibition in tube formation in HUVECs through a unique $AT_{1–7}$ receptor that is sensitive to losartan.

Treatment of HUVECs with the AT_2 receptor antagonist PD123319 had no effect on the Ang-(1–7) mediated inhibition of endothelial tube growth. Although some studies have previously reported that the activation of the AT_2 receptor can lead to an inhibition in growth [50–52], no effect of the AT_2 receptor antagonist was observed on the Ang-(1–7)-induced decrease in tube formation in HUVECs. In addition, Ang-(1–7) has a very poor affinity for AT_2 receptor binding sites [45, 53]. Therefore, the inhibition of tube formation by Ang-(1–7) cannot be explained by the activation of AT_2 receptors.

While no studies have previously been done to investigate the effects of Ang-(1–7) on tube formation during pregnancy, our findings of the inhibition of tube formation in HUVECs by Ang-(1–7) indicate a potential role for Ang-(1–7) in placental vascularization and growth during pregnancy. Previous studies showed that components of the RAS are present in the uteroplacental unit during pregnancy including prorenin, angiotensinogen, ACE, Ang II, and AT_1 receptors [54–56]. In addition, one study described the presence of a non-AT_1/non-AT_2 receptor, which could potentially be hypothesized as the AT_{1-7} receptor, in the trophoblast cells of the human placenta [55]. Our laboratory was the first to definitively show that Ang-(1–7) and its generating enzyme, ACE2, can be found in the placenta during pregnancy [21]. However, the role of Ang-(1–7) in the placenta during pregnancy is largely unknown. Previous studies done in our lab showed that human plasma and urinary levels of Ang-(1–7) increased during the third trimester of normal pregnancy [7, 8]. In addition, studies done in normal pregnant Sprague–Dawley rats showed that Ang-(1–7) is increased in both the kidney and urine [57]. Our findings in this study, that Ang-(1–7) inhibits tube formation in HUVECs, provides evidence that Ang-(1–7) may be important in counteracting the angiogenic effects of Ang II. Thus, the balance of Ang II to Ang-(1–7) in the placenta may be responsible for regulating vascular growth during normal pregnancy. Due to these findings, more studies focused on investigating the role of Ang-(1–7) on the regulation of angiogenesis in the uteroplacental unit during pregnancy are warranted.

In conclusion, these studies demonstrate that Ang-(1–7) causes an inhibition in HUVEC tube formation and growth. The Ang-(1–7) mediated inhibition of endothelial cell tube formation was blocked by treatment with either A779 or losartan. Our studies provide no evidence for an effect of AT_2 receptors in the decrease in tube formation in HUVECs. These results suggest that Ang-(1–7) has an anti-angiogenic effect on HUVECs through a unique AT_{1-7} receptor that is sensitive to A779 and losartan. Our results indicate that the anti-growth actions of Ang-(1–7) could be counter regulating the growth enhancing actions of Ang II. Thus, in abnormal pregnancies, the balance of Ang II to Ang-(1–7) could be skewed toward Ang-(1–7) initiating a state of decreased angiogenesis in the placenta and thus resulting in a decrease of maternal placental blood flow and a reduction in fetal health.

Materials and methods

Cell culture

HUVECs were purchased from Cambrex Bioscience (Walkersville, MD) and were maintained in endothelial cell growth medium 2 (EGM-2) purchased from Cambrex Bioscience (Walkersville, MD) containing growth factors (R^3-IGF, rhVEGF, rhFGF-B, rhEGF), 2% fetal bovine serum (FBS), 50 μg/ml gentamicin, and 50 μg/ml amphotericin. HUVECs were seeded in T-75 cm^2 tissue culture flasks and grown in complete EGM-2 media until confluent. Confluent HUVECs grown in passages three or four were used for the in vitro angiogenesis assay described next.

In vitro angiogenesis assay

In vitro angiogenesis assay kits purchased from Chemicon International (Temecula, CA) were used. A 96-well plate was coated with diluted endothelial cell matrix (ECMatrix), a basement membrane consisting of laminin, collagen type IV, heparan sulfate, proteoglycans, entactin, nidogen, growth factors, and various proteolytic enzymes. HUVECs were then seeded onto the solidified ECMatrix at a concentration of 8×10^3–1×10^4 cells per well in 150 μl of EGM-2 media. The cells were then treated with or without varying concentrations of Ang-(1–7) (Bachem, King of Prussia, PA) (0.17 nM–17 μM) ($n = 5$) or a combination of Ang-(1–7) (1.7 μM) and angiotensin receptor antagonists, 17 μM dose of D-[Ala^7]-Ang-(1–7) [A779 or D-Ala], an AT_{1-7} receptor antagonist, and losartan or PD123319, AT_1 and AT_2 receptor antagonists respectively ($n = 7$–10). Experiments were also conducted in cells treated with each of the inhibitors alone ($n = 8$–10). For each experiment, control vehicle-treated cells were plated in quadruplicate wells while treated cells were plated in duplicate wells. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 18 h to allow endothelial cell tube structures to grow. After 18 h, photographs of four fields in each well were taken with a camera attached to a light microscope. The photographs were then magnified in Photoshop (Adobe Systems, San Jose, CA) for analysis. The points where the endothelial cell tubes intersect (branch points) were manually counted by placing red dots at each intersection point (Fig. 1a). The number of branch points in the four quadrants in each well were averaged and the mean number of branch points in either four (control) or two (treatments) wells were averaged to give the final result of an overall average of the number of branch points for each treatment. Branch points were counted by a person blinded to the treatment type.

Statistics and data analysis

Data were analyzed with a standard one way analysis of variance (ANOVA) followed by the Newman Keul's post hoc test. The Student's *t* test for unpaired observations was

used when appropriate (GraphPad Software, San Diego, CA). A P value of less than 0.05 was considered statistically different. All arithmetic means are presented ± SEM.

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