

RESEARCH ARTICLE

Caffeine treatment disturbs the angiogenesis of zebrafish embryos

Chien-Hung Yeh¹, Yun-Feng Liao², Chao-Yuan Chang³, Jen-Ning Tsai⁴, Yun-Hsin Wang⁵, Chien-Chung Cheng⁶, Chi-Chung Wen⁷, and Yau-Hung Chen^{1,2}

¹Graduate Institute of Life Sciences, Tamkang University, Tamsui, New Taipei City, Taiwan, ²Department of Chemistry, Tamkang University, Tamsui, New Taipei City, Taiwan, ³Department of Chemical and Materials Engineering, Tamkang University, Tamsui, New Taipei City, Taiwan, ⁴School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung, Taiwan, ⁵Division of Basic Research, Koo Foundation Sun Yat-Sen Cancer Center, Taipei, Taiwan, ⁶Department of Applied Chemistry, National Chia-Yi University, Chia-Yi, Taiwan, and ⁷Department of Mathematics, Tamkang University, New Taipei City, Taiwan

Abstract

Caffeine is a widely consumed substance that occurs in numerous dietary sources, but teratogenic effects of caffeine intake during embryonic development are still not clear. In the present study, we used the zebrafish as a model to assess caffeine-induced toxicity on embryonic vascular development. A green fluorescent vascular endothelium transgenic line, Tg(fli1:egfp), was utilized for the sensitive detection of vascular development, including vasculo- and angiogenesis. Caffeine-treated embryos showed no defects in vasculogenesis, but revealed dose-dependent (250–350 ppm) developmental defects in intersegmental vessels, dorsal longitudinal anastomotic vessels, and subintestinal vein sprouting. Further, real-time polymerase chain reaction analysis of caffeine-treated embryos showed an upregulation of *nrp1a* along with a downregulation of *sema3aa* and *sema3c*. In conclusion, caffeine treatment induces defects of angiogenesis in zebrafish embryos.

Keywords: Angiogenesis, caffeine, fluorescent protein, transgenic fish, zebrafish

Introduction

Caffeine (1,3,7-trimethylxanthine) is a widely consumed purine alkaloid that occurs in numerous dietary sources, such as tea, coffee, and soft drinks. Although the U.S. Food and Drug Administration lists caffeine as a generally recognized safe food substance with multiple purposes, overuse of caffeine may have adverse effects, especially for pregnant women. In humans, caffeine can pass through the placenta to the fetus. There is growing, but conflicting, evidence demonstrating that maternal caffeine intake can have adverse effects on fetal development (Browne, 2006; Bakker et al., 2010; Brent et al., 2011). For example, pregnant women with higher caffeine intake have been reported to be associated with a higher risk of spontaneous abortion, late miscarriage, and stillbirth (Christian and Brent, 2001). In rats, caffeine has been reported to

induce cardiovascular malformations (Matsuoka et al., 1987). In zebrafish, caffeine treatment led to some neuromuscular defects and, consequently, caused movement disorder (Chen et al., 2008). These observations indicated that caffeine has potential embryotoxic and teratogenic effects on human and other animal species.

In medical applications, caffeine has been reported to interact with ryanodine receptors (RyRs) and adenosine receptors (ADRs) (Tsai and Barish, 1995; Huang et al., 2005). RyRs and ADRs are abundant in muscles as well as the nervous and cardiovascular systems; they play roles in regulating intracellular calcium release, coronary blood flow, and angiogenesis (Fredholm et al., 1997). Although we will be focusing on the potential harmful effects of caffeine, other studies have noted therapeutic

Address for Correspondence: Yau-Hung Chen, Department of Chemistry, Tamkang University, 151 Ying-chuan Road, Tamsui, New Taipei City 25137, Taiwan; Fax: 886-2-2620-9924; E-mail: yauhung@mail.tku.edu.tw

(Received 09 August 2011; revised 10 September 2011; accepted 13 September 2011)

uses of caffeine through interaction with the RyR and ADR receptors (Jacobson and Gao, 2006).

Angiogenesis is a process in which new capillaries are formed by sprouting from the primary vascular plexus (Risau, 1997). Angiogenesis is conserved between species and occurs not only during embryogenesis, but also during pathogenesis of adult organs. Several animal models have been utilized to assay angiogenesis, such as chick chorioallantoic membrane, Matrigel plug, corneal micro-pocket assay, and zebrafish embryos (Norrby, 2006). The primary vasculature in the zebrafish, as in other vertebrates, is generated by vasculogenesis (i.e., the formation of blood vessels from endothelial precursor cells called angioblasts). Trunk circulation of zebrafish embryo begins at approximately 24–26 hpf (Fouquet et al., 1997; Isogai et al., 2001). Subsequent sprouting and branching of blood vessels is formed by angiogenesis (i.e., the formation of new blood vessels from preexisting vessels). During embryonic angiogenesis, the intersegmental vessels (Se) are subsequently formed by angiogenic sprouting from dorsal aorta to the dorsal side of the trunk, and mature Se consequently form dorsal longitudinal anatomic vessels (DLAVs).

In this study, we used the zebrafish model to assess the caffeine-induced effects on vascular development (especially on angiogenesis). The subtle changes of vascular development were easily observed by using the green-fluorescent-vascular endothelium line, Tg(*flil:egfp*) (Lawson and Weinstein, 2002), as well as using histochemical staining of alkaline phosphatase (ALP) activity. Further, the molecular mechanisms underlying the effects of caffeine on vascular development were also investigated.

Methods

Zebrafish embryos

Tg(*flil:egfp*) zebrafish were obtained from the Zebrafish International Resource Center, University of Oregon (Eugene, Oregon, USA) (Lawson and Weinstein, 2002). Both wild-type (WT; AB strain) and Tg(*flil:egfp*) zebrafish were kept under a 14-hour light and 10-hour dark photoperiod at 28.5°C. The procedures for zebrafish culture, embryo collection, and fluorescence observation have been described previously (Chen et al., 2007). Designation of zebrafish developmental stages was done by following the standard criteria (Kimmel et al., 1995). All animal experiments in this study were performed in accord with the guidelines issued by the regional animal ethic committee.

Caffeine exposure

Caffeine (C₈H₁₀N₄O₂; Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in sterile, distilled water to the desired concentrations. Three caffeine exposure methods were designed using different exposure onsets and durations (method I: 0–24 hpf; method II: 12–36 hpf; and method III: 12–60 hpf). For dose-titration experiments, zebrafish

embryos were collected and randomly divided into the desired groups. Each group was cultivated in 24-well microplates and exposed to either water (mock-control) or water containing caffeine of the desired concentrations (250 and 350 ppm). All embryos were counted for the number of somites, as well as fully connected DLAVs at the exposure checkpoint (24, 36, or 60 hpf).

Quantification of DLAV growth

Zebrafish embryos were anesthetized by 0.006% tricaine (Sigma-Aldrich) and photographed under a fluorescence stereomicroscope (Leica Microsystems Inc., Buffalo Grove, New York, USA), equipped with a green fluorescent protein filter set (Chroma Technology Corp., Bellows Falls, Vermont, USA). Acquired images were analyzed using Image-Pro Plus software (Media Cybernetics Inc., Bethesda, Maryland, USA). To get a better quantitative view of how caffeine affected DLAVs, we defined “DLAV number” in this study. In brief, DLAV number means the number of somites containing fully connected DLAVs. In this study, a field comprising 10 somites of each embryo, in both anterior and posterior portions relative to the caudal end of the yolk extension (Figure 1A), was photographed at multiple focal planes. The number of somites containing fully connected DLAVs (i.e., DLAV number) was subsequently counted on both lateral sides of each embryo (i.e., 20 somites were examined per embryo).

Whole-mount ALP staining

Endogenous ALP activity of WT embryos were stained according to standard procedures (Habeck et al., 2002; Serbedzija et al., 1999). In brief, embryos were fixed at 3 dpf in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 minutes. Fixed embryos were dehydrated in methanol, permeabilized in acetone (30 minutes at –20°C), and washed in PBST (PBS with 0.1% Tween-20) twice. Embryos were equilibrated in staining buffer (0.1 M of Tris-HCl [pH 9.5], 50 mM of MgCl₂, 0.1 M of NaCl, and 0.1% Tween-20) for 45 minutes. Staining reaction was started by adding 2.25 μL of nitro blue tetrazolium (NBT) and 1.75 μL of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) per mL of staining buffer for 15–30 minutes (stock solutions: 75 mg/mL of NBT in 70% dimethylformamide; and 50 mg/mL of BCIP in dimethylformamide), then stopped by adding PBST.

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNAs from each group of zebrafish embryos were extracted and reverse-transcribed by using the standard procedure, as described previously (Chen et al., 2009; Wang et al., 2009). Gene-specific PCR primer sequences are listed in Table 1. qPCR was performed by the following conditions: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C, using 2× Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, USA) and 200 nM of forward and reverse gene-specific primers (Table 1). Each assay was

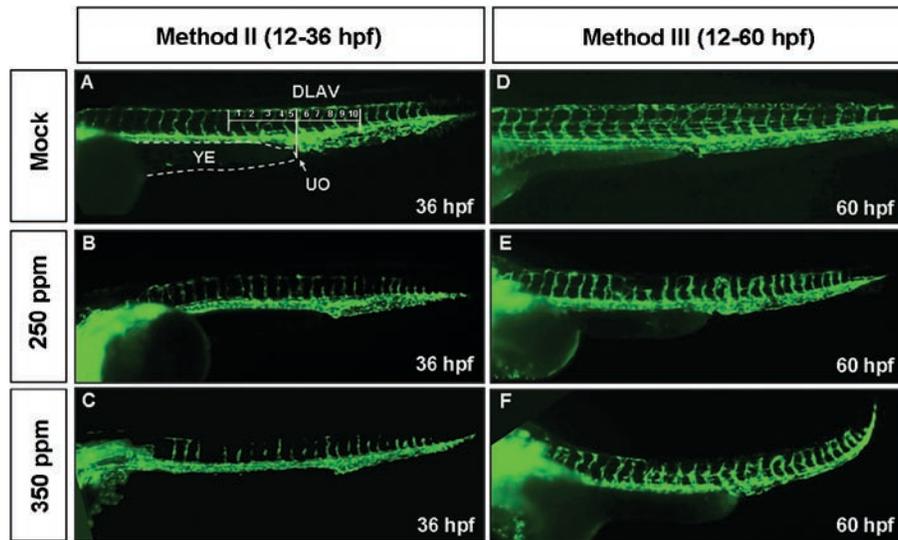


Figure 1. Development of DLAVs in caffeine-treated *Tg(fli1:egfp)* embryos. Embryos were treated with caffeine (0, 250, or 350 ppm) using method II (12–36 hpf) or III (12–60 hpf). Images of each embryo were acquired at the developmental stage of 36 or 60 hpf. YE, yolk extension; UO, urogenital opening.

Table 1. Primers used in this study.

Primer	Sequence (5' → 3')
β -actin-F	CAGCAAGCAGGAGTACGATGAGT
β -actin-R	TTGAATCTCATTGCTAGGCCATT
<i>Sema3aa</i> -F	GAACGCATCCCAAAGGAGTACA
<i>Sema3aa</i> -R	AAATCAGATCCCAATCGGAGC
<i>Sema3c</i> -F	GCCATGGACTACAGGATCCTTT
<i>Sema3c</i> -R	CCTCGACCTTACTGATGGACG
<i>nrp1a</i> -F	GGAAATCCATTCGGACCAAA
<i>nrp1a</i> -R	AGGCGTCCAGCCGTTCTC

run in triplicate on a 7300 real-time PCR system (Applied Biosystems). Relative fold changes of expression were derived using the comparative C_T method, and β -actin was used as an endogenous control.

Statistical analysis

All analyses in this study were carried out by JMP statistical software (version 4.02; SAS Institute, Inc., Cary, North Carolina, USA). Dosage and exposure-time effects were examined by a generalized linear regression model. A P -value less than 0.05 was considered statistically significant in all analyses.

Results

To investigate the effect of caffeine on vasculo- and angiogenesis during early embryonic development, we treated transgenic *Tg(fli1:egfp)* zebrafish with caffeine using different exposure methods (method I: 0–24 hpf; method II: 12–36 hpf; and method III: 12–60 hpf). The vasculogenesis of zebrafish embryos was not affected after caffeine exposure at early stages (method I: 0–24 hpf; data not shown), but the development of Se and DLAVs was disturbed in caffeine-treated embryos at later stages (Figure 1; method II: 12–36 hpf; method III:

12–60 hpf). The vascular development of embryos was thus examined at 36 and 60 hpf. Compared with mock control (0 ppm of caffeine; Figure 2A and D), caffeine disturbed the formation of Se in the somite boundaries (Figure 2B, C, E, and 2F). The angiogenic sprouting from dorsal aorta to the dorsal side of the trunk was prematurely stalled upon caffeine treatment, leading to incomplete formation of the Se.

Not only Se formation was disturbed, but caffeine also significantly affected DLAV development. Thus, we defined “DLAV number” to get a better quantitative view for studying how caffeine affected DLAV formation. To study dosage and time effects of caffeine exposure on DLAV number, we treated zebrafish embryos with three dosages, including 0, 250, and 300 ppm under two exposure protocols (method II: 12–36 hpf; method III: 12–60 hpf). The number of embryos tested in each dosage group for methods II and III were 60 and 50, respectively. We first depicted, in Figure 2, the mean number of DLAVs with its standard error (SE) for each dosage level under two exposure methods. It can be observed, in Figure 3, that the average DLAV numbers decreased significantly from 19.42 ± 0.13 (mean \pm SE) and 8.18 ± 0.30 to 4.65 ± 0.28 when embryos were treated with 0, 250, and 350 ppm of caffeine under method II. A similar phenomenon was also observed from method III: Approximately 19.62 ± 0.12 , 8.38 ± 0.37 , and 3.64 ± 0.36 DLAV numbers arose for 0, 250, and 350 ppm. However, no apparent difference in average DLAV number was observed for each dosage level between exposure methods. Next, we used generalized linear regression analysis to examine the dosage and exposure-time effect on DLAV number. Results from JMP showed that the dosage and exposure-time effects were -4.40 (SE = 0.08; $P < 0.0001$) and -0.20 (SE = 0.23; $P = 0.3701$) respectively.

The former significantly indicates approximately 4.40 DLAV number decreases when one more 100 ppm of concentration increases, whereas the latter means no significant difference in DLAV number between two exposure methods.

To confirm whether the effect of caffeine was direct or indirect (e.g., muscle development), we observed the development of the subintestinal vein (SIV) in caffeine-treated embryos (Figure 3). After being treated with caffeine (250 and 350 ppm, respectively) from 12 to 60 hpf, the SIV of each embryo was observed at 72 hpf. The SIV basket with fully connected lumen appeared in the mock-control embryos (Figure 3A). After caffeine treatment, the SIV basket with fully connected lumen was strongly reduced or absent (Figure 3B). Interrupted and reduced branches of SIV spanning the yolk were observed in caffeine-treated embryos. Consistently, such caffeine-induced defects in SIV formation were also revealed in WT embryos using ALP staining (Figure 3C and D).

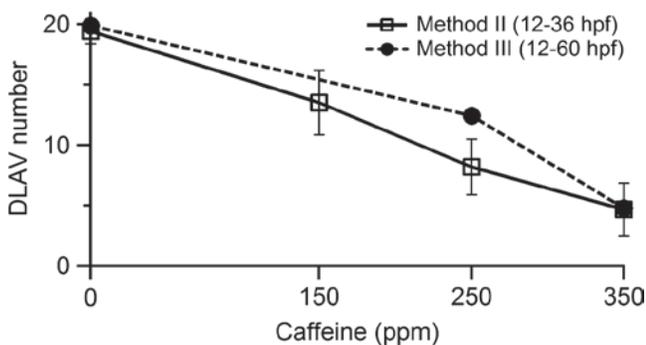


Figure 2. Number of DLAVs (DLAV number) in caffeine-treated Tg(*flil:egfp*) embryos. Embryos were treated with caffeine (0, 150, 250, or 350 ppm) using (A) method II (12–36 hpf) or (B) method III (12–60 hpf). DLAV number of each embryo was counted at the developmental stage of 36 or 60 hpf.

The molecular mechanism of caffeine-induced defects in angiogenesis was further investigated by real-time PCR in the caffeine-treated groups (Figure 4). We examined three target genes involved in vertebrate blood vessel formation: neuropilin 1a (*nrp1a*), semaphoring 3aa (*sema3aa*), and semaphoring 3c (*sema3c*). Nrp1a is a coreceptor for vascular endothelial growth factor receptor and Plexin. Nrp1a/Plexin is the receptor of semaphoring 3A (SEMA3A), which is considered to be a proangiogenic factor, whereas SEMA3C has been shown to have antiangiogenesis properties (Gu et al., 2003; Moriya et al., 2010). The messenger RNA (mRNA) level of *nrp1a* in the caffeine-treated group was significantly increased to 6.42 ($P < 0.05$) by fold change, compared to the corresponding mock-control group. On the other hand, mRNA levels of *sema3aa* and *sema3c* in the caffeine-treated group were significantly decreased to 0.44 ($P < 0.05$) and 0.45 ($P < 0.05$) by fold change, compared to the corresponding mock-control group. These observations revealed that caffeine affected angiogenesis through mediation of the factor for proangiogenic factors (*sema3aa* and *sema3c*) and coreceptor (*nrp1a*). These regulatory mechanisms may explain why the caffeine-induced defects in angiogenic sprouting could be partially restored after the interruption of caffeine treatment.

Discussion

In this study, we found that a dosage of 250–350 ppm of caffeine is able to disturb the angiogenesis of zebrafish embryos. In this regard, comparison of the caffeine dose between this study (250–350 ppm) and dietary intake is an important issue that should be discussed. The average weight of each zebrafish embryo is ~1.2 mg. After appropriate calculation, 250–350 ppm of caffeine is estimated to be 4,200–5,800 mg/kg (Chen et al., 2008). This range is much higher than the acceptable daily intake of caffeine in adult people (150–300 mg per day) (Christian and Brent,

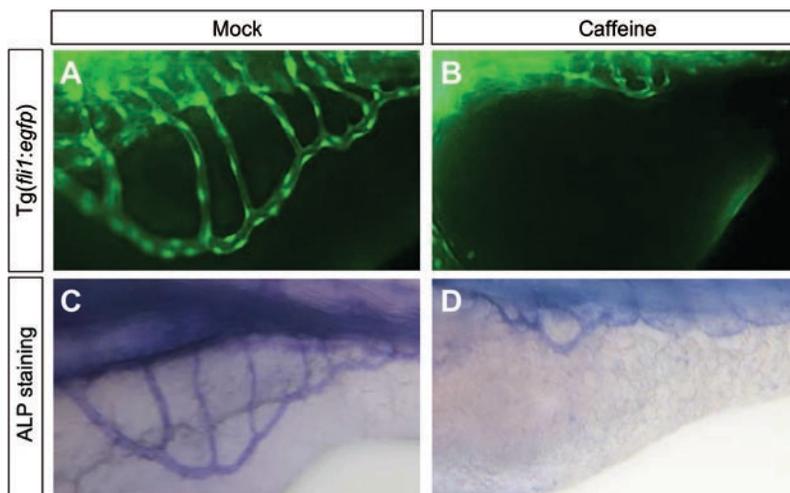


Figure 3. Effects of caffeine on the formation of the SIV. (A) SIV of Tg(*flil:egfp*) embryos. (B) caffeine-treated. (C and D) ALP staining of WT embryos: (C) no-treatment control versus (D) with caffeine treatment. Caffeine-treated embryos were exposed to 350 ppm of caffeine from 12 to 60 hpf. All embryos were observed at the developmental stage of 72 hpf.

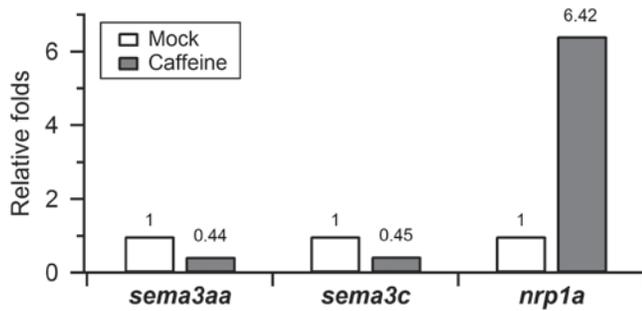


Figure 4. Relative quantification of mRNA expression using the comparative C_T method (C_T : cycles of qPCR; relative folds to control group = $2^{-\Delta\Delta C_T}$; the caffeine-treated group is significantly different from the corresponding mock group).

2001), but very close to the doses (250–8,100 mg) that were used to study caffeine-induced toxicities in other animal models (Nolen, 1981). Thus, we suggest that daily intake of caffeine should not cause angiogenesis defects.

From the molecular aspect, caffeine acts as an antagonist against ADRs. There is growing evidence that ADRs play an important role in angiogenesis and wound healing (Feoktistov et al., 2009; Fredholm, 2010; Jacobson and Gao, 2006). It is reported that activation of the ADRs promotes angiogenesis by increasing the release of angiogenic factors (Linden, 2005). Moreover, the caffeine-induced defects in angiogenesis were partially restored after the interruption of caffeine treatment, suggesting that such defects may lead to the release of angiogenic factors to overcome the disturbance from caffeine.

Conclusion

In conclusion, our results revealed that caffeine influences vascular development in zebrafish embryos, including defects in Se, DLAV, and SIV. Further, we have implicated the roles of *nrp1a*, *sema3aa*, and *sema3c* in mediating the antiangiogenesis effects of caffeine. This could provide novel insights into the subtle changes induced by caffeine, which are worthy of further tests, in higher vertebrates.

Declaration of interest

This project was supported by the National Science Council, Republic of China (grant no.: NSC 96-2313-B-032-001-MY3).

References

Bakker, R., Steegers, E. A. P., Obradov, A., Raat, H., Hofman, A., Jaddoe, V. W. V. (2010). Maternal caffeine intake from coffee and tea, fetal growth, and the risks of adverse birth outcomes: the Generation R Study. *Am J Clin Nutr* 91:1691–1698.

Brent, R. L., Christian, M. S., Diener, R. M. (2011). Evaluation of the reproductive and developmental risks of caffeine. *Birth Defects Res B* 92:152–187.

Browne, M. (2006). Maternal exposure to caffeine and risk of congenital anomalies: a systematic review. *Epidemiology* 17:324–331.

Chen, Y. H., Chen, W. L., Wang, Y. H., Huang, M. Y., Chern, M. K. (2007). Spatiotemporal expression of zebrafish D-amino acid oxidase during early embryogenesis. *Fish Physiol Biochem* 33:73–80.

Chen, Y. H., Huang, Y. H., Wen, C. C., Wang, Y. H., Chen, W. L., Chen, L. C., et al. (2008). Movement disorder and neuromuscular change in zebrafish embryos after exposure to caffeine. *Neurotoxicol Teratol* 30:440–447.

Chen, Y. H., Lin, Y. T., Lee, G. H. (2009). Novel and unexpected functions of zebrafish CCAAT box binding transcription factor (NF-Y) B subunit during cartilages development. *Bone* 44:777–784.

Christian, M. S., Brent, B. L. (2001). Teratogen update: evaluation of the reproductive and developmental risks of caffeine. *Teratology* 64:51–78.

Feoktistov, I., Biaggioni, I., Cronstein, B. N. (2009). Adenosine receptors in wound healing, fibrosis and angiogenesis. In: Wilson, C. N., Mustafa, S. J. (Eds.), *Adenosine receptors in health and disease*. Handbook of experimental pharmacology (vol. 193, pp. 383–397). Berlin Heidelberg: Springer Verlag.

Fouquet, B., Weinstein, B. M., Serluca, F. C., Fishman, M. C. (1997). Vessel patterning in the embryo of the zebrafish: guidance by notochord. *Dev Biol* 183:37–48.

Fredholm, B. B., Abbracchio, M. P., Burnstock, G., Dubyak, G. R., Harden, T. K., Jacobson, K. A., et al. (1997). Towards a revised nomenclature for P1 and P2 receptors. *Trends Pharmacol Sci* 18:79–82.

Fredholm, B. (2010). Adenosine receptors as drug targets. *Exp Cell Res* 316:1284–1288.

Gu, C., Rodriguez, E. R., Reimert, D. V., Shu, T., Fritzsche, B., Richards, L. J., et al. (2003). Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev Cell* 5:45–57.

Habeck, H., Odenthal, J., Walderich, B., Maischein, H. M. (2002). Analysis of a zebrafish VEGF receptor mutant reveals specific disruption of angiogenesis. *Curr Biol* 12:1405–1412.

Huang, ZL, Qu, WM, Eguchi, N, Chen, JF, Schwarzschild, MA, Fredholm, BB, et al. (2005). Adenosine A2A, but not A1, receptors mediate the arousal effect of caffeine. *Nat Neurosci* 8:858–859.

Isogai, S., Horiguchi, M., Weinstein, B. M. (2001). The vascular anatomy of the developing zebrafish: an atlas of embryonic and early larval development. *Dev Biol* 230:278–301.

Jacobson, K. A., Gao, Z. G. (2006). Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov* 5:247–264.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev Dyn* 203:253–310.

Lawson, N. D., Weinstein, B. M. (2002). *In vivo* imaging of embryonic vascular development using transgenic zebrafish. *Dev Biol* 248:307–318.

Linden, J. (2005). Adenosine in tissue protection and tissue regeneration. *Mol Pharmacol* 67:1385–1387.

Matsuoka, R., Uno, H., Tanaka, H., Kerr, C. S., Nakazawa, K., Nadal-Ginard, B., et al. (1987). Caffeine induces cardiac and other malformations in the rat. *Am J Med Genet* 28:433–443.

Moriya, J., Minamino, T., Tateno, K., Okada, S., Uemura, A., Shimizu, I, et al. (2010). Inhibition of semaphorin as a novel strategy for therapeutic angiogenesis. *Circ Res* 106:391–398.

Nolen, G. A. (1981). The effect of brewed and instant coffee on reproduction and teratogenesis in the rat. *Toxicol Appl Pharmacol* 58:171–183.

Norrby, K. (2006). *In vivo* models of angiogenesis. *J Cell Mol Med* 10:588–612.

Risau, W. (1997). Mechanisms of angiogenesis. *Nature* 386:671–674.

Serbedzija, G. N., Flynn, E., Willett, C. E. (1999). Zebrafish angiogenesis: a new model for drug screening. *Angiogenesis* 3:353–359.

Tsai, T. D., Barish, M. E. (1995). Imaging of caffeine-inducible release of intracellular calcium in cultured embryonic mouse telencephalic neurons. *J Neurobiol* 27:252–265.

Wang, Y. H., Wen, C. C., Yang, Z. S., Cheng, C. C., Tsai, J. N., Ku, C. C., et al. (2009). Development of a whole-organism model to screen new compounds for sun protection. *Mar Biotechnol (NY)* 11:419–429.