

## Original Article

# Does Folic Acid Supplementation Rescue Defects in ECE-1-Deficient Mouse Embryos?

(endothelin-converting enzyme / mice / endothelin 1 / cardiac malformations / endothelin receptor)

A. HAQUE<sup>1</sup>, B. ŠAŇKOVÁ<sup>1,2</sup>, A. KVASILOVÁ<sup>1</sup>, E. KREJČÍ<sup>1,2</sup>, D. SEDMERA<sup>1,2</sup>

<sup>1</sup>Institute of Anatomy, First Faculty of Medicine, Charles University in Prague, Czech Republic

<sup>2</sup>Department of Cardiovascular Morphogenesis, Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

**Abstract.** Endothelin (ET) signalling is essential for normal embryonic development. Disruption of this pathway leads to defects in the development of subsets of cranial and cephalic neural crest derivatives. Endothelin-converting enzyme 1 (ECE-1) is a rate-limiting step in the biosynthesis of ET-1. Recently, there has been considerable interest in the protective role of folic acid (FA) against congenital anomalies via increasing the expression of ET-1. We have tested whether FA supplementation can rescue craniofacial and cardiac defects observed in the *ECE1*<sup>-/-</sup> embryos. *ECE1*<sup>+/-</sup> mice were caged together to obtain litters containing embryos of all possible genotypes. The treatment group had the diet supplemented with 20 mg/kg of FA from the day of discovery of the vaginal plug. FA supplementation did not result in modified proportions of the genotypes, indicating no rescue of the embryonic mortality. There was also no effect on the litter size. Craniofacial and cardiac defects were likewise identical in the *ECE1*<sup>-/-</sup> embryos of both groups. There was a mild but significant reduction in the embryo size in wild-type and heterozygous FA-supplemented embryos, and there were haemorrhages in the wild-type supplemented embryos at ED14.5. Expression of ET receptor A detected by immunohistochemistry was up-regulated in

the *ECE1*<sup>-/-</sup> embryos, but FA supplementation had no effects on the distribution of staining intensity. We conclude that FA is not able to rescue the phenotype in this model, suggesting an alternative pathway for its action. These results also caution against indiscriminate use of dietary supplements in attempts to prevent congenital anomalies.

## Introduction

The role of endothelin 1 (ET-1) as a mediator of intercellular signalling for normal embryonic development is well known (Kurihara et al., 1994), and the function of the ET signalling cascade is conserved among vertebrates (Clouthier and Schilling, 2004). ET-1 is also a potent vasoconstrictor, paracrine signalling molecule with mitogenic activity. It is the most abundantly and widely expressed member of the endothelin family (Yanagisawa et al., 1988). It is proteolytically generated from its inactive precursor by ECE-1. Transcription of the *edn1* gene yields a 212-aa, preproET-1, which undergoes a multi-step cleavage process. Furin-like protease converts it into an inactive intermediate, 38-aa big ET-1, which acts as a substrate for endothelin-converting enzymes. The end result is in the form of active 21-aa ET-1, capable of acting on endothelin receptor (ETR) A. Genetic disruption of this ET/ECE-1/ETRA pathway leads to defects in the development of subsets of cranial and cephalic neural crest derivatives including branchial arch-derived craniofacial tissues as well as cardiac outflow and great vessel structures (Clouthier et al., 1998).

Endothelins exist as three isopeptides, ET-1, ET-2, and ET-3. They are encoded by different loci and act on two distinct G-protein-coupled heptahelical receptors called ETRA and ETRB (Arai et al., 1990; Sakurai et al., 1990). These receptors have distinct affinities (Levin, 1995). ETRA exclusively binds ET-1 and ET-2, while ETRB binds all three forms of endothelins with equal affinity.

The role of ET-1 in the development of neural crest-derived structures has been a focus of many recent studies. Arising from the midbrain/hindbrain region, the neural crest cells carry some positional information as they undergo epithelial to mesenchymal transition and populate

Received March 4, 2014. Accepted April 18, 2014.

The project was supported by the Grant Agency of the Czech Republic P302/11/1308, 13-12412S, Ministry of Education, Youth and Sports of the Czech Republic, PRVOUK P35/LF1/5, and Academy of Sciences RVO: 67985823. Barbora Šaňková was also supported by a SVV training grant from Charles University in Prague.

Corresponding author: David Sedmera, Institute of Anatomy, First Faculty of Medicine, Charles University in Prague, U Nemocnice 3, 128 00, Prague 2, Czech Republic. Phone: (+420) 224 965 941; Fax: (+420) 224 965 770; e-mail: david.sedmera@lf1.cuni.cz

Abbreviations: BSA – bovine serum albumin, ECE – endothelin-converting enzyme, ED – embryonic day, ET – endothelin, ETR – endothelin receptor, FA – folic acid, PBS – phosphate-buffered saline, PCR – polymerase chain reaction, PFA – paraformaldehyde.

the branchial arches. In the arches their patterning is dependent on signalling molecules including ET-1. ET-1 is expressed in the disto-caudal pharyngeal arch ectoderm, the core paraxial mesoderm and the pharyngeal pouch endoderm of the mandibular portion of the first arch as well as in arches 2–6 (Maemura et al., 1996; Clouthier et al., 1998). ET-1/ETRA signalling is required for normal craniofacial and cardiac development in both cell-autonomous and non-autonomous manner (Clouthier et al., 2003).

ET-1 has long been implicated in the complex interplay that occurs between intrinsic genes and extrinsic environmental signals during morphogenesis. Thomas et al. (1998) suggested a model in which epithelial secretion of ET-1 stimulates mesenchymal expression of dHAND, which regulates *Msx1* expression in the growing distal branchial arch. Complete disruption of this molecular pathway results in growth failure of the branchial arches from excess apoptosis, while partial disruption leads to defects of branchial arch derivatives, similar to those seen in the CATCH 22 syndrome. Interestingly, a recent population genetics study identified polymorphisms in the human *ECE1* gene as a susceptibility factor for sporadic congenital heart disease, specifically tetralogy of Fallot and perimembranous ventricular septal defect (Wang et al., 2012).

Endothelins play an indispensable role in the development of neural crest derivatives. They act via two independent signalling pathways (ET-1/ETRA and ET-3/ETRB), affecting different subsets of neural crest lineages. The phenotype observed in *ET1*<sup>-/-</sup> (Kurihara et al., 1994) or *ETRA*<sup>-/-</sup> (Clouthier et al., 1998) embryos does not overlap with that in *ET3*<sup>-/-</sup> (Baynash et al., 1994) or *ETRB*<sup>-/-</sup> (Hosoda et al., 1994) mice, indicating that the cell populations affected in *ET1*<sup>-/-</sup> or *ETRA*<sup>-/-</sup> embryos are different from those in *ET3*<sup>-/-</sup> or *ETRB*<sup>-/-</sup> mice. The mice deficient for ET-1 or ETRA show defects in development of craniofacial structures and great vessels. The homozygous null mice are not viable and die at birth mainly due to mechanical asphyxia and cardiac abnormalities. The phenotype resembles a spectrum of human conditions collectively termed as CATCH 22 (Wilson et al., 1993), velo-cardio-facial syndrome (Shprintzen et al., 1978; Goldberg et al., 1993). ET-3/ETRB mice show spotted coat colour and aganglionic megacolon due to developmental defects in melanocytes and enteric neurons, respectively (Baynash et al., 1994; Hosoda et al., 1994). ET-2 mRNA is expressed exclusively in the post-natal gastrointestinal tract in mice.

ECE-1 is a physiologically relevant rate-limiting enzyme for conversion of both big ET-1 and big ET-3 *in vivo*. *ECE1*<sup>-/-</sup> mice (Yanagisawa et al., 1998b) reproduced the phenotype resulting from the defects in both ET-1/ETRA and ET-3/ETRB-mediated signalling pathways. *ECE1*<sup>-/-</sup> embryos showed embryonic lethality apparently due to cardiac failure, which was not observed in any of the single knockouts of ET-related molecules, i.e., ET-1, ET-2, ET-3, ETRA, ETRB and ECE-2. The unique role of ECE-1 in cardiac development is de-

scribed to be due to its ability to cleave both big ET-1 and big ET-3 *in vivo*.

ECE-1 is thus a key enzyme in the biosynthesis of ET-1. *ECE1*<sup>-/-</sup> embryos exhibit craniofacial and cardiac defects, which are virtually identical to the defects seen in ET-1 and ETRA deficient embryos. Although ECE-1 is predominant, it is not the exclusive enzyme involved in the generation of ETs (D'Orleans-Juste et al., 2003). It has been reported that large amounts of mature ET-1 peptides are found in *ECE1*<sup>-/-</sup> mice. Other moieties have been suggested to be involved in the genesis of ETs besides ECE-1, including the possibility of transplacental transfer of ETs.

Null mutation in the *ECE2* gene in mice showed no detectable developmental defects (Yanagisawa et al., 2000). This indicates that ECE-2 is a redundant protease for the activation of big ETs *in vivo*. However, studies show that the phenotype of *ECE1* null embryos worsens on an *ECE2* null background. This genetic interaction of the *ECE1* and *ECE2* genes suggests that ECE-2 also participates in the formation of active ET-1 *in vivo*, indicating cooperation between ECE-1 and ECE-2 (Yanagisawa et al., 2000).

Recently, there has been considerable interest in the protective role of folic acid (FA) against congenital anomalies via increasing the expression of ET-1. It was shown by immunohistochemistry and whole-mount *in situ* hybridization that FA significantly increased the mRNA expression of ET-1 in the first branchial arch (Zhang et al., 2006). The mechanism by which FA imparts its effects on ET-1 expression is not clear and is still under investigation. According to authors FA might be increasing the concentration of signalling factors by donating its methyl group to methionine synthase, pivotal to DNA synthesis, or it might be acting through increasing the numbers of FOLR1 receptors.

This study was designed to investigate the potential of FA to rescue the developmental phenotype in *ECE1*<sup>-/-</sup> mice. Since mature endothelins are detected in considerable amounts in *ECE1*<sup>-/-</sup> mice, there is a possibility that the protective effect of FA, if any, would be due to its actions on factors independent of ECE-1.

## Material and Methods

### Knockout mice

The colony of *ECE1*<sup>-/-</sup> mice (Yanagisawa et al., 1998b) was generated from founders provided by Dr Yanagisawa (University of Texas Southwestern Medical Center, Dallas, TX). These mice were bred to a mixed Swiss background and were maintained as heterozygous breeding pairs. The mice were housed in a standard animal facility, with normal 12-h light/dark cycle. The heterozygous pairs were mated overnight. Pregnancy was confirmed by examining the vaginal plug in the morning of the following day, noon of which was designed as embryonic day (ED) 0.5. Female mice were then housed in individual cages and divided into two groups. Group 1

was given FA supplemented diet (20 mg/kg diet, SEMED, Prague, Czech Republic), while the control group was fed standard mouse chow. Based on general values for consumption and weight (Han et al., 2009; Marean et al., 2011), these mice were ingesting 80 micrograms of FA per day while on supplementation. Pregnant mice were sacrificed by cervical dislocation on ED 12.5 and ED 14.5. Embryos were rapidly dissected out of uterine horns and separated from the placenta in phosphate-buffered saline (PBS) solution. Gross morphology of individual viable embryos and their placentae was examined. Embryos were digitally photographed under an Olympus SZX (Olympus, Prague, Czech Republic) dissecting microscope against a ruler to assess body dimensions.

We studied 64 embryos at ED 12.5 and 78 embryos at ED 14.5. Experimental protocols met the research guidelines established in accordance with the Act on Animal Protection of the Czech Republic (311/1997).

### Genotyping

Genomic DNA was prepared from tail biopsies of embryos using DirectPCR(Tail) (Viagen Biotech Inc., Los Angeles, CA) and subjected to polymerase chain reaction (PCR) with RedTaq Ready Mix (Sigma-Aldrich, St. Louis, MO) using primer sets described previously (Yanagisawa et al., 1998a).

### Histological analysis

Embryos harvested at ED12.5 and ED14.5 were fixed overnight in 4% paraformaldehyde (PFA) solution in PBS on ice with gentle rocking. Samples were then dehydrated in a series of ethanols, followed by clearing in xylene, and then embedded in paraplast. Continuous 7  $\mu$ m sections of the entire embryos were cut in series. Every other section was stained with Mayer's haematoxylin and eosin. The embryos were examined for heart defects.

### Immunohistochemical examination

*ECE1* and wild-type (WT) mice were collected at ED 12.5 and 14.5 (three per genotype at each gestational age), fixed in 4% PFA in PBS overnight followed by ethanol dehydration, clearing in xylene and paraplast

embedding. Primary antibody used for immunohistochemistry was anti-ETRA (polyclonal rabbit antibody, 1 : 200, Abcam, Cambridge, UK). The staining was performed in dark humid chamber. Deparaffinized sections were heat-treated twice for 5 min in citrate buffer (pH 6.0), and then blocked in normal goat serum (1 : 10) and in 1% bovine serum albumin (BSA) in PBS with 0.1% Triton-X (Sigma-Aldrich) for 60 min at room temperature. Primary antibody was then applied overnight at +4 °C. On day 2, the sections were washed in three changes of PBS and TRITC-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, PA) was applied for 4 h in the dark at room temperature. Negative controls were obtained by omission of the primary antibody. After washing in PBS, the nuclei were counterstained with Hoechst 33258 (1 : 100,000 diluted in 0.1% Triton-X in distilled water, Sigma-Aldrich #861405). In the end the sections were washed with distilled water and dehydrated in an ascending series of ethanol (70%–100%), cleared in xylene and mounted in Depex permanent medium (Electron Microscopy Sciences, Hatfield, PA).

Images were acquired in an Olympus BX51 fluorescence microscope in visible and appropriate epifluorescence illumination using an Olympus DP71 CCD camera. Plates were then assembled in Adobe Photoshop. Digital image processing involved background subtraction, level adjustment, and Unsharp Mask filtering.

### Statistical analysis

All results are expressed as means  $\pm$  SD. The comparison between groups was performed using either unpaired *t*-test (continuous variable – crown rump length), or  $\chi^2$  (survival of embryos). All statistical analyses were performed using Microsoft Excel. A *P* value of less than 0.05 was considered significant.

## Results

Embryonic size and percentage of genotypes recovered at different days of gestation are summarized in Table 1. In a separate set of experiments, the survival of

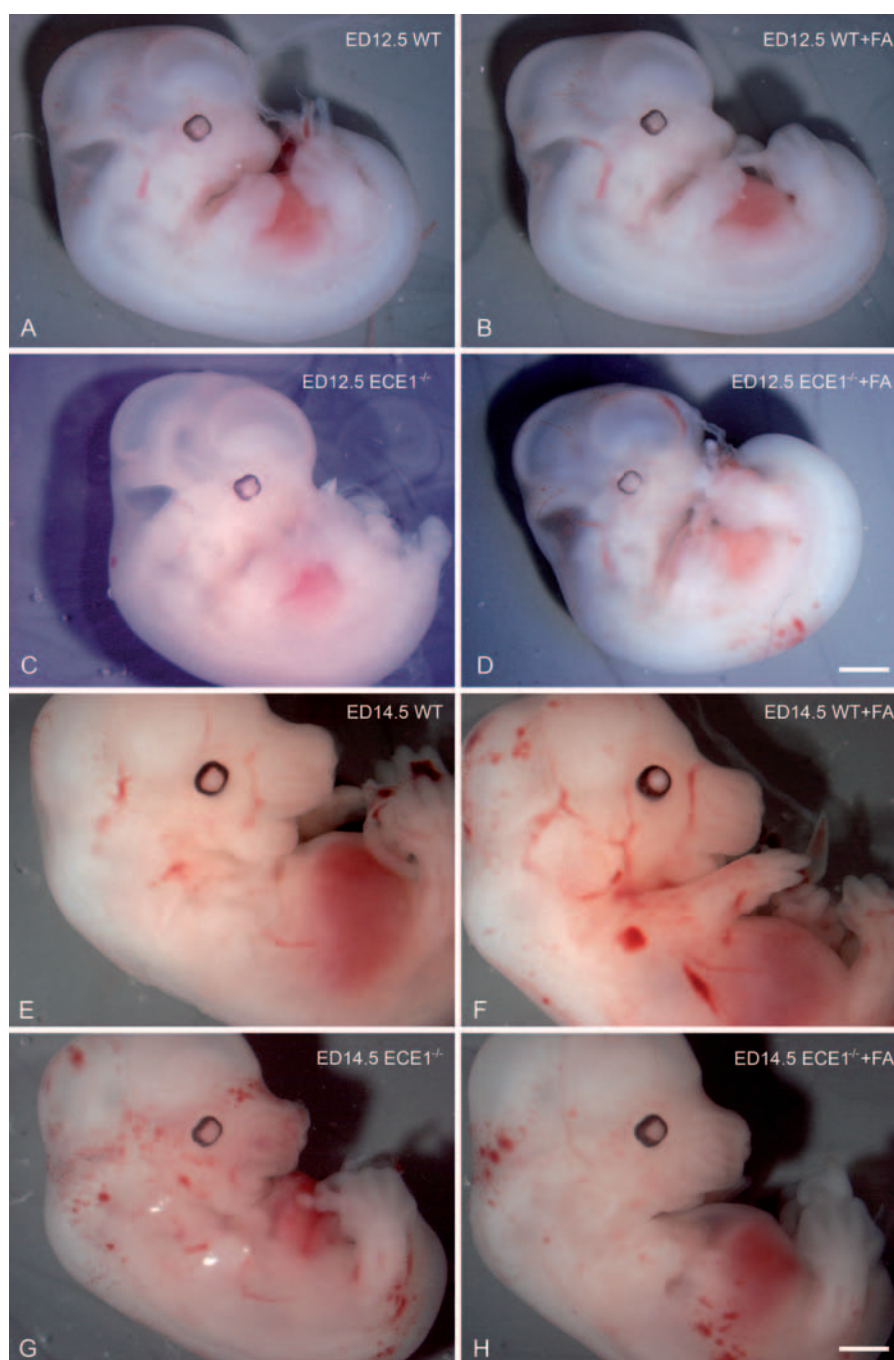
*Table 1. Survival and embryonic size in mouse embryos with and without folic acid (FA) supplementation. The recovery of null embryos was lower than the expected 25 % (Pearson's  $\chi^2$ , *P* = NS) at both stages analysed, suggesting a negative selection against them (Yanagisawa et al., 1998b); they were also 2–3 % smaller than their littermates (\**P* < 0.05). There was no significant effect of FA supplementation on null embryo survival; however, the wild-type and heterozygous supplemented embryos tended to be slightly smaller (+*P* < 0.05) than their untreated counterparts. Values are mean  $\pm$  SD.*

Genotype	ED12.5	%	CRL	ED14.5	%	CRL
WT	9	29	8.23 $\pm$ 0.05	10	42	10.22 $\pm$ 0.07
WT + FA	8	30	8.09 $\pm$ 0.06 <sup>+</sup>	8	29	10.10 $\pm$ 0.07 <sup>+</sup>
ECE +/-	17	55	8.32 $\pm$ 0.13	11	46	10.24 $\pm$ 0.05
ECE +/- + FA	14	52	8.09 $\pm$ 0.06 <sup>+</sup>	18	64	10.11 $\pm$ 0.07 <sup>+</sup>
ECE -/-	5	16	8.06 $\pm$ 0.09*	3	12	10.06 $\pm$ 0.05*
ECE -/- + FA	5	18	7.98 $\pm$ 0.08*	2	7	10.10 $\pm$ 0.00
<b>Total N control</b>	<b>31</b>	<b>100</b>		<b>24</b>	<b>100</b>	
<b>Total N FA</b>	<b>27</b>	<b>100</b>		<b>28</b>	<b>100</b>	

*ECE1*<sup>-/-</sup> mutants at ED 15.5 and 16.5 was also observed at a small percentage, in contrast to the original report on this line (Yanagisawa et al., 1998a). One dead *ECE1*<sup>-/-</sup> neonate was also recovered during the course of the experiments. There was a trend towards the proportion of mutant embryos in the litter lower than the expected 25 % (16 % at ED12.5, 12 % at ED14.5), but the difference did not reach statistical significance due to relatively small numbers. FA supplementation did not significantly influence the survival at any stage analysed (Table 1). The mutant embryos showed oedema and

haemorrhages (Fig. 1), and were slightly smaller than both wild-type and heterozygous littermates. FA supplementation led to a small decrease in embryo size in all wild-type and heterozygous, but not *ECE1*<sup>-/-</sup> embryos (Table 1), while the number of embryos per litter did not show a significant difference between the control and FA-supplemented group (total average for controls: 9 embryos per litter, range 5–12; FA, 8 per litter, range 4–11).

The phenotype of *ECE1*<sup>-/-</sup> mutant embryos at ED14.5 depicted in Figure 1 showed, in addition to haemor-



*Fig. 1.* External morphology of mouse embryos in different treatment groups. Note that at ED12.5, the mutant embryos appear smaller than wild-type littermates, and show occasional haemorrhages; there is no noticeable effect of FA supplementation. The mutants at ED14.5 show clearly shortened mandible, a phenotype not rescued by FA; in addition, skin haemorrhages are also seen in wild-type embryos with FA supplementation. Scale bar 1 mm.

rhages, distinctly shorter mandible, allowing their clear identification. There was no difference in phenotype between control and FA-supplemented mutants; the other genotypes looked normal in both groups (Fig. 1).

Since it is believed that the mortality in this mouse line is linked to heart malformations, we examined the serial sections through the thorax to ascertain the cardiac phenotype (Fig. 2). In mutant embryos, there was always present a ventricular septal defect, apparent already at ED12.5 as enlarged interventricular foramen, and FA supplementation had no influence on this phenotype. Similarly, there were no differences between control and FA-supplemented mutant embryos at ED14.5 (data not shown).

To gain a better insight into the consequences of perturbation of the ET signalling pathway by deletion of its rate-limiting biosynthesis enzyme, we performed immunohistochemical analysis of its major receptor, ETRA.

We observed positive staining in numerous regions of the embryo, in particular the neural tube, dorsal root ganglia, and skin (Fig. 3). The staining in the heart was stronger in the non-myocytes, specifically in the subepicardium and subendocardium. It was distinctly stronger in the mutants (Fig. 3). FA treatment did not have any influence on the staining patterns or intensity (Fig. 3 and data not shown).

## Discussion

The ET signalling pathway has multiple essential roles in embryonic development, and targeted deletion of its components results in similar but distinct phenotypes (Kurihara et al., 1994; Clouthier et al., 1998; Yanagisawa et al., 2000). Usage of this signalling cascade seems to be conserved across species (Clouthier and Schilling, 2004), as also evidenced by mimicking

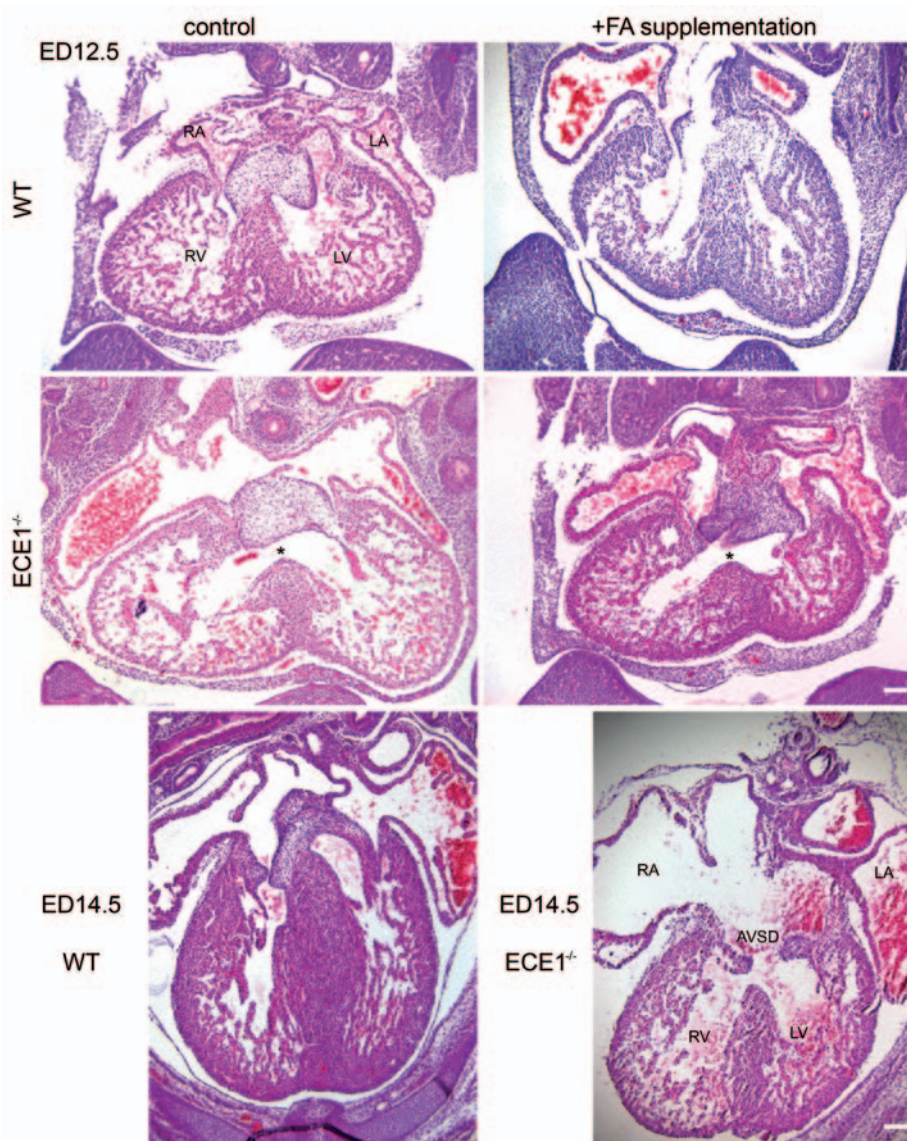


Fig. 2. Heart defects in *ECE1*<sup>-/-</sup> mice are not rescued by FA treatment. The mutants at ED12.5 show already deficient ventricular septation manifested as enlarged interventricular foramen (\*) and some thinning of the ventricular wall. The mutant phenotype of deficient atrioventricular septation (AVSD) and ventricular wall thinning is clear at ED14.5. Scale bars 50  $\mu$ m.

the phenotype by pharmacological receptor blockade in the chick (Kempf et al., 1998; Sedmera et al., 2008). Our observations in the *ECE1*<sup>-/-</sup> mouse model correspond with the original description of the phenotype (Yanagisawa et al., 1998a, 2000), the only difference being occasional survival of the embryos past ED14.5 that could be ascribed to different genetic background (we bred the mice to mixed Swiss background for purposes of a different study).

In our analysis, we focused on the craniofacial and cardiovascular phenotype, and did not observe any influence of FA supplementation on either of these. Considering the mechanism of action of FA, this could be explained by the protective action of FA through its receptors that is independent of ET signalling. The lack of prevention by FA in our study could very well be attributed to its signalling path different from and independent of that responsible for affecting the expression of endothelins. Folate is known to act via increasing the expression of folate responsive genes through folate re-

ceptors. Gene methylation is regarded as the possible mechanism for enhancing folate responsive genes including those of the Notch signalling pathway, which is critical in neural crest development (Evans et al., 2008; Zhang et al., 2008).

The dose of FA used in this study was fairly high, ten times higher than the dose recommended to humans in attempt to prevent neural tube defects (Wald et al., 2001). In agreement with previous data (Mikael et al., 2013) reporting developmental toxicity of the same dose of FA (20 mg/kg, 10× higher than the recommended amount), we observed slight reduction in the size of embryos in the supplemented group, which cannot be explained by increased litter size since the average number of embryos per litter was actually smaller. However, we did not see increased foetal loss, ventricular septal defects or wall thinning in non-mutant embryos; this could be due to variation of the diet composition or sensitivity of the particular mouse strain. A similar study (Pickell et al., 2011) also showed negative effects of

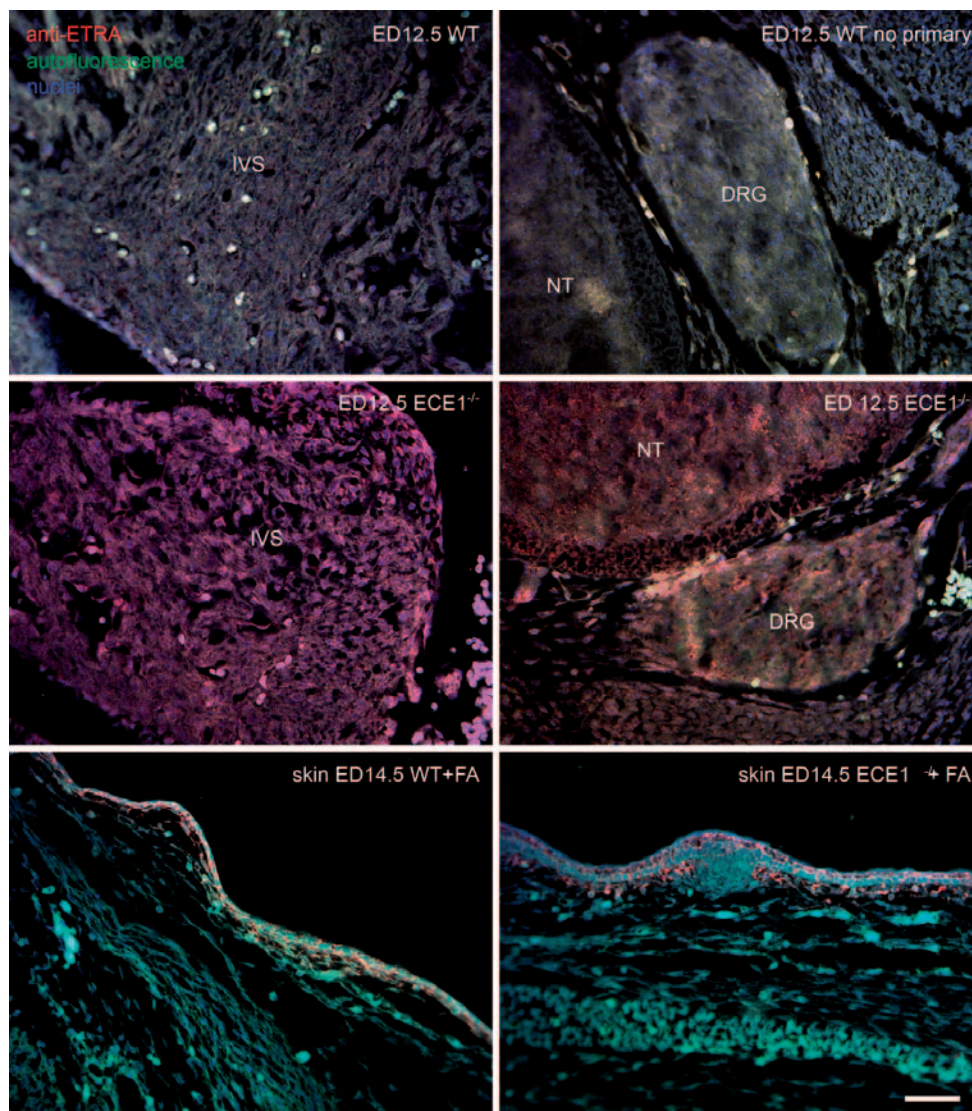


Fig. 3. Expression of endothelin receptor A in mouse embryonic tissues. Immunohistochemical analysis showed increased expression in the mutant interventricular septum; there was also abundant staining in the neural tissues. There was no difference in staining extent or intensity with the FA supplementation, as exemplified in the chest wall skin. Scale bar 10  $\mu$ m.

high FA doses in mice, which could be “rescued” by maternal methylene tetrahydrofolate reductase deficiency.

The interaction between the genotype and the environment in pathogenesis of congenital malformations is well recognized and could account for a considerable proportion of cases of congenital anomalies. Classical examples include wild variation of sensitivity of various strains of chick embryos to ethanol (Bruyere et al., 1994); some of the toxicity could be rescued by vitamin C or FA (Memon and Pratten, 2009). However, dietary supplements should not be regarded as a panacea, especially in cases of well-defined genetic defects using an unrelated pathway, as seems to be the case in our model. This corresponds well with a significant drop, but not complete elimination, of human neural tube defects coincident with introduction of FA-fortified food in the United States (Honein et al., 2001; Hesecker et al., 2009; Osterhues et al., 2013). While our study did not show any rescue effect of FA in a well-defined genetic mouse model, it would be interesting to compare this with a human population study of sporadic forms of congenital heart disease (Wang et al., 2012), where such an effect remains a possibility.

In conclusion, our study shows that while there is room for epigenetic modifications of certain genetically determined phenotypes, dietary supplementation is not capable of rescuing all the defects and could have some undesirable side effects. There is need for further studies of admittedly complex interactions between the genetic makeup and environment in order to customize recommendations to pregnant women for optimal pregnancy outcomes.

### Acknowledgement

Our sincere thanks are due to Yanagisawa Lab for their generous gift of the *ECE1<sup>-/-</sup>* mice. We would like to thank Prof. Miloš Grim for his continued support of this project and critical reading of the manuscript.

### References

- Arai, H., Hori, S., Aramori, I., Ohkubo, H., Nakanishi, S. (1990) Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* **348**, 730-732.
- Baynash, A. G., Hosoda, K., Giaid, A., Richardson, J. A., Emoto, N., Hammer, R. E., Yanagisawa, M. (1994) Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. *Cell* **79**, 1277-1285.
- Bruyere, H. J. Jr., Stith, C. E., Thorn, T. A. (1994) Cardio-teratogenic dose of ethanol reduces both lactic dehydrogenase and succinic dehydrogenase activity in the bulbar ridges of the embryonic chick heart. *J. Appl. Toxicol.* **14**, 27-31.
- Clouthier, D. E., Hosoda, K., Richardson, J. A., Williams, S. C., Yanagisawa, H., Kuwaki, T., Kumada, M., Hammer, R. E., Yanagisawa, M. (1998) Cranial and cardiac neural crest defects in endothelin-A receptor-deficient mice. *Development* **125**, 813-824.
- Clouthier, D. E., Williams, S. C., Hammer, R. E., Richardson, J. A., Yanagisawa, M. (2003) Cell-autonomous and nonautonomous actions of endothelin-A receptor signaling in craniofacial and cardiovascular development. *Dev. Biol.* **261**, 506-519.
- Clouthier, D. E., Schilling, T. F. (2004) Understanding endothelin-1 function during craniofacial development in the mouse and zebrafish. *Birth Defects Res. C Embryo Today* **72**, 190-199.
- D’Orleans-Juste, P., Plante, M., Honore, J. C., Carrier, E., Labonte, J. (2003) Synthesis and degradation of endothelin-1. *Can. J. Physiol. Pharmacol.* **81**, 503-510.
- Evans, C. O., Yao, C., Laborde, D., Oyesiku, N. M. (2008) Folate receptor expression in pituitary adenomas: cellular and molecular analysis. *Vitam. Horm.* **79**, 235-266.
- Goldberg, R., Motzkin, B., Marion, R., Scambler, P. J., Shprintzen, R. J. (1993) Velo-cardio-facial syndrome: a review of 120 patients. *Am. J. Med. Genet.* **45**, 313-319.
- Han, M., Serrano, M. C., Lastra-Vicente, R., Brinez, P., Acharya, G., Huhta, J. C., Chen, R., Linask, K. K. (2009) Folate rescues lithium-, homocysteine- and Wnt3A-induced vertebrate cardiac anomalies. *Dis. Model. Mech.* **2**, 467-478.
- Hesecker, H. B., Mason, J. B., Selhub, J., Rosenberg, I. H., Jacques, P. F. (2009) Not all cases of neural-tube defect can be prevented by increasing the intake of folic acid. *Br. J. Nutr.* **102**, 173-180.
- Honein, M. A., Paulozzi, L. J., Mathews, T. J., Erickson, J. D., Wong, L. Y. (2001) Impact of folic acid fortification of the US food supply on the occurrence of neural tube defects. *JAMA* **285**, 2981-2986.
- Hosoda, K., Hammer, R. E., Richardson, J. A., Baynash, A. G., Chung, J. C., Giaid, A., Yanagisawa, M. (1994) Targeted and natural (piebald-lethal) mutations of endothelin-B receptor gene produce megacolon associated with spotted coat color in mice. *Cell* **79**, 1267-1276.
- Kempf, H., Linares, C., Corvol, P., Gasc, J. M. (1998) Pharmacological inactivation of the endothelin type A receptor in the early chick embryo: a model of mispatterning of the branchial arch derivatives. *Development* **125**, 4931-4941.
- Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W. H., Kamada, N., Jishage, K., Ouchi, Y., Azuma, S., Toyoda, Y., Ishikawa, T., Kumada, M., Yazaki, Y. (1994) Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature* **368**, 703-710.
- Levin, E. R. (1995) Endothelins. *N. Engl. J. Med.* **333**, 356-363.
- Maemura, K., Kurihara, H., Kurihara, Y., Oda, H., Ishikawa, T., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Yazaki, Y. (1996) Sequence analysis, chromosomal location, and developmental expression of the mouse preproendothelin-1 gene. *Genomics* **31**, 177-184.
- Marean, A., Graf, A., Zhang, Y., Niswander, L. (2011) Folic acid supplementation can adversely affect murine neural tube closure and embryonic survival. *Hum. Mol. Genet.* **20**, 3678-3683.
- Memon, S., Pratten, M. K. (2009) Developmental toxicity of ethanol in chick heart in ovo and in micromass culture can

- be prevented by addition of vitamin C and folic acid. *Reprod. Toxicol.* **28**, 262-269.
- Mikael, L. G., Deng, L., Paul, L., Selhub, J., Rozen, R. (2013) Moderately high intake of folic acid has a negative impact on mouse embryonic development. *Birth Defects Res. A Clin. Mol. Teratol.* **97**, 47-52.
- Osterhues, A., Ali, N. S., Michels, K. B. (2013) The role of folic acid fortification in neural tube defects: a review. *Crit. Rev. Food Sci. Nutr.* **53**, 1180-1190.
- Pickell, L., Brown, K., Li, D., Wang, X. L., Deng, L., Wu, Q., Selhub, J., Luo, L., Jerome-Majewska, L., Rozen, R. (2011) High intake of folic acid disrupts embryonic development in mice. *Birth Defects Res. A Clin. Mol. Teratol.* **91**, 8-19.
- Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K., Masaki, T. (1990) Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* **348**, 732-735.
- Sedmera, D., Harris, B. S., Grant, E., Zhang, N., Jourdan, J., Kurkova, D., Gourdie, R. G. (2008) Cardiac expression patterns of endothelin-converting enzyme (ECE): implications for conduction system development. *Dev. Dyn.* **237**, 1746-1753.
- Shprintzen, R. J., Goldberg, R. B., Lewin, M. L., Sidoti, E. J., Berkman, M. D., Argamaso, R. V., Young, D. (1978) A new syndrome involving cleft palate, cardiac anomalies, typical facies, and learning disabilities: velo-cardio-facial syndrome. *Cleft Palate J.* **1**, 56-62.
- Thomas, T., Kurihara, H., Yamagishi, H., Kurihara, Y., Yazaki, Y., Olson, E. N., Srivastava, D. (1998) A signaling cascade involving endothelin-1, dHAND and msx1 regulates development of neural-crest-derived branchial arch mesenchyme. *Development* **125**, 3005-3014.
- Wald, N. J., Law, M. R., Morris, J. K., Wald, D. S. (2001) Quantifying the effect of folic acid. *Lancet* **358**, 2069-2073.
- Wang, Y., Liu, Y., Peng, W., Wang, M., Sun, J., Zhang, Z., Mo, X. (2012) ECE1 polymorphisms may contribute to the susceptibility of sporadic congenital heart disease in a Chinese population. *DNA Cell Biol.* **31**, 1425-1430.
- Wilson, D. I., Burn, J., Scambler, P., Goodship, J. (1993) DiGeorge syndrome: part of CATCH 22. *J. Med. Genet.* **30**, 852-856.
- Yanagisawa, H., Hammer, R. E., Richardson, J. A., Williams, S. C., Clouthier, D. E., Yanagisawa, M. (1998a) Role of Endothelin-1/Endothelin-A receptor-mediated signaling pathway in the aortic arch patterning in mice. *J. Clin. Invest.* **102**, 22-33.
- Yanagisawa, H., Yanagisawa, M., Kapur, R. P., Richardson, J. A., Williams, S. C., Clouthier, D. E., de Wit, D., Emoto, N., Hammer, R. E. (1998b) Dual genetic pathways of endothelin-mediated intercellular signaling revealed by targeted disruption of endothelin converting enzyme-1 gene. *Development* **125**, 825-836.
- Yanagisawa, H., Hammer, R. E., Richardson, J. A., Emoto, N., Williams, S. C., Takeda, S., Clouthier, D. E., Yanagisawa, M. (2000) Disruption of ECE-1 and ECE-2 reveals a role for endothelin-converting enzyme-2 in murine cardiac development. *J. Clin. Invest.* **105**, 1373-1382.
- Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., Masaki, T. (1988) A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* **33**, 411-415.
- Zhang, X., Liu, H., Cong, G., Tian, Z., Ren, D., Wilson, J. X., Huang, G. (2008) Effects of folate on notch signaling and cell proliferation in neural stem cells of neonatal rats in vitro. *J. Nutr. Sci. Vitaminol. (Tokyo)* **54**, 353-356.
- Zhang, Z., Xu, Y., Li, L., Han, J., Zheng, L., Liu, P., Li, Y. (2006) Prevention of retinoic acid-induced early craniofacial abnormalities by folinic acid and expression of endothelin-1/dHAND in the branchial arches in mouse. *Br. J. Nutr.* **96**, 418-425.