Identification of Semi-dominant Phenotypes within *Zic2*$^{Ku/+}$ embryos 14.5 days post coitum

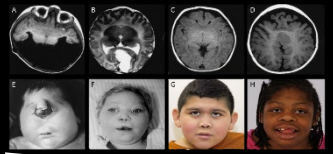
Kyle A. Drover[1], Zoe M. Thomas[1], Kristen S. Barratt[1], Neil R. Horner[2], Henrik Westerberg[2], Ruth M. Arkell[1]

1. Early Mammalian Development Laboratory, John Curtin School of Medical Research, The Australian National University, Canberra, ACT, Australia
2. Medical Research Council Harwell Institute, Harwell OX11 0RD, UK

Introduction

What is Holoprosencephaly? Adult form and function are dependent upon the activity of specialised signalling centres that act early in development at the embryonic midline. These centres instruct the surrounding cells to adopt a positional fate and to form the patterned structures of the phylotypic embryo[1]. Abnormalities in these processes have devastating consequences for the individual, as exemplified by holoprosencephaly (HPE) in which anterior midline development fails, leading to structural defects of the brain and/or face (**Fig. 1**) [2,3].

Figure 1: Human Holoprosencephaly is a spectrum of brain and craniofacial defects. HPE severity is a spectrum that ranges from severe (no hemisphere division and cyclopia) to mild (a subtle facial phenotype in the absence of structural brain abnormalities).



HPE severity

Human HPE: Although rare at birth (prevalence: 1:10,000)[4], HPE is estimated to occur in 1:250 human conceptuses[5], indicating that the low neonatal frequency is a consequence of a high rate of HPE-induced embryonic lethality and suggesting HPE is a common disorder. Consistent with this, non-syndromic HPE exhibits autosomal dominant inheritance with incomplete penetrance and variable expressivity. The majority of solved human cases with normal chromosomes have a pathogenic mutation in one of four HPE driver genes (*ZIC2*, *SHH*, *FGF8* or *SIX3*).[6]

The mouse as a model for HPE research: Mutations in each orthologous murine gene also causes HPE. However, the ability of the mouse to model human HPE genetic architecture has been called into question since human cases are overwhelmingly associated with dominant inheritance, whereas in the mouse, phenotypes are generally only found in the homozygous state. This is true of the Kumba mouse model of *Zic2*-associated HPE, in which (on the C3H/HeH congenic background) homozygous embryos are found to have a severe form of HPE (**Fig. 2**)[7]. whereas heterozygotes are indistinguishable from their wildtype littermates [8,9]

Figure 2: The Kumba (*Ku*) model of *Zic2* associated HPE. Homozygous mutant embryos exhibit the most severe form of HPE (known as cyclopia) with a single eye (e) and a proboscis (p).

Aims

We hypothesize that the apparent human-mouse dosage discrepancy is due to differences in the way human and mouse populations are studied. For example, heterozygotes may have subtle phenotypes not normally detected in the assays typically employed in mouse studies, and murine phenotypes may have been analysed on strains (inbred or outbred) that are relatively resistant to HPE. We have addressed this hypothesis with two Aims:

- Aim 1. Use sophisticated and systemic phenotyping to determine if *Zic2*$^{Ku/+}$ embryos on the C3H/HeH background have subtle phenotypes.
- Aim 2. Establish a colony of *Zic2*$^{Ku/+}$ animals congenic on the HPE permissive C57BL/6N background and determine if heterozygotes exhibit HPE.

Methods

Mouse Husbandry:

The kumba (*Ku*) allele of *Zic2*[6,10] was maintained on two distinct backgrounds by continuous backcross to either C3H/HeH or C57BL6/N mice. For the production of staged embryos, 12 P.M. on the day of the appearance of the vaginal plug is designated 0.5 dpc.

Sample Preparation for µCT scanning via PTA staining:

Embryos were dissected at 14.5 dpc and a PTA-based staining protocol was used to provide soft tissue contrast. Briefly, embryos were rinsed 5 times with MilliQ water and then placed in 2.5% PTA within a decreasing ethanol series (70% : 7 days, 50% : 24 hrs, 30% 24 hrs) and finally 2.5% PTA in 4% PFA, with the solutions changed daily. µCT scanning was performed on a Caliper Quantum FX micro-CT using the maximal source voltage (90kV), source current (200uA) and a field-of-view of 20 (voxel resolution of 40µm). After scanning, the reconstructions (i.e. the scans) were saved as DICOM (.dcm) files with a resolution of 512x512x512 voxels.

Volumetric Analysis via LAMA:

DICOM files were converted into NRRD files using custom scripts designed within the Arkell lab and then analysed via LAMA (as described in [11]) but with a custom protocol suitable for scans obtained from the Quantum FX µCT machine.

Results

LAMA analysis shows that *Zic2*$^{Ku/+}$ embryos on a C3H/HeH background have reduced lateral ventricle volume (a phenotype indicative of HPE).

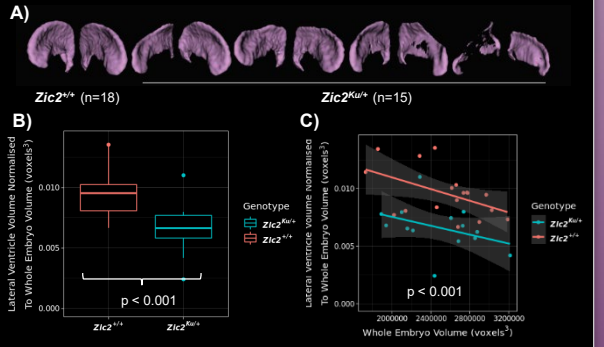


Figure 3, *Zic2*$^{Ku/+}$ embryos on the C3H/HeH background have reduced lateral ventricle volume at 14.5 dpc. A) Visual representations of the lateral ventricles of wild-type and *Zic2*$^{Ku/+}$ C3H/HeH embryos 14.5 dpc (posterior to anterior view). As shown, the lateral ventricles of *Zic2*$^{Ku/+}$ embryos range from being similar to wild-type (left) to severely reduced compared to wild-type (right). Boxplots B) and regressions C) are shown of the lateral ventricle volume normalised to whole embryo volume (WEV) as described in Horner *et al 2021*. p value obtained from a linear model normalised and regressed to WEV.

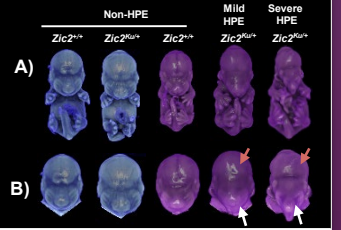
- In addition, LAMA analysis revealed that the mesencephalic vesicles, midbrain, spinal cord and left lobe of the liver are affected in *Zic2*$^{Ku/+}$ embryos.

Table 1. Affected organs detected via LAMA

Affected organ	Organ System	P-value (3dp)
Lateral Ventricle	nervous system	4.70
Mesencephalic Vesicle	nervous system	0.040
Midbrain	nervous system	0.027
Spinal Cord Marginal Layer	nervous system	7.47×10^{-4}
Left Lobe of Liver	alimentary system	0.040

This semi-dominant phenotype is exacerbated on the C57BL6/N HPE permissive genetic background (Fig. 4)

Figure 4, *Zic2*$^{Ku/+}$ embryos on the C57BL6/N background have exacerbated HPE at 14.5 dpc. Volume renderings of A) the full body and B) the head of C3H/HeH and C57BL6/N embryos (blue and purple respectively). *Zic2*$^{Ku/+}$ C57BL6 embryos display narrowing of the craniofacial region (white arrow) and the cerebral hemispheres (red arrow)



Conclusions

In conclusion, the use of the sophisticated analysis software (LAMA) allowed the identification of a subtle phenotype within Zic2$^{Ku/+}$ embryos that is heavily influenced by the genetic background. This provides evidence that HPE within both man and mouse is semi-dominant and influenced by genetic modifiers.

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