Kumar, A., Davies, T. G., & Itasaki, N. (2018). Developmental abnormalities of the otic capsule and inner ear following application of prolyl-hydroxylase inhibitors in chick embryos. *Birth Defects Research*, *110*(15), 1194-1204. https://doi.org/10.1002/bdr2.1375

Developmental abnormalities of the otic capsule and inner ear following application of prolyl-hydroxylase inhibitors in chick embryos

Akshay Kumar[1], Thomas G. Davies[2] and Nobue Itasaki[1*]

[1]Faculty of Health Sciences, University of Bristol, Bristol BS2 8EJ, UK
[2]School of Earth Sciences, University of Bristol, Bristol BS8 1TQ, UK

Running title: PHD inhibitors on otic capsule

*Correspondence to:
Nobue Itasaki, Faculty of Health Sciences, University of Bristol, Bristol BS2 8EJ, UK
Tel: +44 1179289818
E-mail:nobue.itasaki@bristol.ac.uk

Abstract

Background

Naturally hypoxic conditions in amniote embryos play important roles in normal development. We previously showed that a hypoxic condition is required to produce a sufficient amount of neural crest cells (NCCs) during embryogenesis and that promoting a hypoxic response by prolyl-hydroxylase (PHD) inhibitors increases NCCs. Given that PHD inhibitors are considered as a potential treatment for anaemia and ischemic diseases, we investigated the phenotypic effect of PHD inhibitors on embryonic development.

Methods

Chick embryos were administered with PHD inhibitors prior to the induction of NCCs on day 1.5. Three main events relating to hypoxia, NCCs induction, vasculogenesis and chondrogenesis, were examined.

Results

PHD inhibitors caused an increase of *Sox10*-positive NCCs *in vivo*. Vasculogenesis was promoted temporarily, although rapid vasculogenesis diminished the effect by day 5 in cephalic and pharyngeal regions. Studies on chondrogenesis at day 7 showed advanced development of the otic capsule, a cartilaginous structure encapsulating the inner ear. Analysis by X-ray micro-computed-tomography (μCT) revealed smaller otic capsule, suggesting premature differentiation. This in turn deformed the developing semicircular canals within it. Other skeletal structures such as the palate and jaw were unaffected. The localised effect on the otic capsule was considered a result of the multiple effects from the hypoxic responses, increased NCCs and promoted chondrogenesis.

Conclusion

Given the wide range of clinical applications being considered for PHD inhibitors, this study provides crucial information to caution and guide use of PHD inhibitors when treating women of childbearing age.

Key words

Hypoxia; prolyl-hydroxylase inhibitors; otic capsule; inner ear; semicircular canals; µCT; chick embryos

Introduction

The skeletal structure of the vertebrate head originates from both neural crest cells (NCCs) and head mesoderm (Abzhanov and others, 2007; Couly and others, 1993; Noden and Trainor, 2005; Santagati and Rijli, 2003). A deficit of cranial NCCs may result in congenital craniofacial hypoplasia and facial distortion. Genetic conditions such as Treacher Collins, DiGeorge and CHARGE syndromes and Pierre Robin sequence, as well as non-hereditary conditions such as fetal alcohol syndrome and retinoid embryopathy, are all attributed to NCC defects (Ahlgren and others, 2002; Bajpai and others, 2010; Cartwright and Smith, 1995; Escot and others, 2016; Kiecker, 2016; Rovasio and Battiato, 1995; Tan and others, 2013; Trainor, 2010). NCCs arise from the dorsal neural tube during early embryogenesis by epithelial-mesenchymal transition (EMT) and differentiate not only to neural and pigmental cells but also to facial bones, cartilages and connective tissues among others, providing the skeletal basis for the head and neck structures together with mesodermal cells (Le Douarin and Kalcheim, 1999).

Induction, proliferation, migration and differentiation of NCCs are controlled at multiple steps by a gene regulatory network (Simoes-Costa and Bronner, 2015). In addition to the genetic control, we have recently found that the naturally occurring embryonic hypoxia contributes to the induction of NCCs (Scully and others, 2016), providing a new insight into the mechanism for normal development and new strategies to tackle NCC defects. Under hypoxic conditions, a transcription factor Hypoxia-Inducible Factor (HIF) permits cell adaptation by promoting cell survival, through anaerobic glycolysis and angiogenesis, as well as increased EMT (Semenza, 2014). In adult pathological conditions, this mechanism aids ischaemic tissue recovery, however, it too aids the promotion of cancer progression and metastasis. In normoxia, on the other hand, α-subunit of HIF (HIFα) is hydroxylated at specific Proline residues by oxygen-dependent Prolyl-hydroxylase-domain-containing-enzymes (PHDs), a group of enzymes belonging to 2-oxoglutarate-dependent oxygenases (Bruick and McKnight, 2001; Markolovic and others, 2015). Hydroxylation of HIFα subsequently leads to its ubiquitination and degradation, hence the hypoxic response is blocked (Epstein and others, 2001). Because of this, PHD inhibitors have a great potential in ischemic disease therapies and are under rapid development (Thinnes and others, 2015). While a number of PHD inhibitors are currently in clinical trials (Brigandi and others, 2016; Gupta and Wish, 2017; Provenzano and others, 2016; Yeh and others, 2017), adverse effects such as erythrocytosis and cancer progression are also suggested, due to the role HIFα has in erythropoiesis, angiogenesis, cell survival, invasion and metastasis (McMullin, 2010; Semenza, 2010). As PHD inhibitors may well

be beneficial to pregnant females, for anaemia for example, the effect of the drugs on embryos needs investigation.

Our previous study has shown that embryos exposed to relatively higher oxygen in the *ex ovo* condition shows reduced expression of *Sox9/10* and *Snail2* in the cranial NCCs population, and that application of PHD inhibitors restores it (Scully and others, 2016). The effect of PHD inhibitors was considered as an increase of EMT thus producing a larger amount of NCCs. It was also possible that expression of *Sox9/10* genes, regulators of hypoxic response (Amarilio and others, 2007; Li and others, 2013), was enhanced in individual cells, and/or up-regulation of the HIF pathway by PHD inhibitors resulted in up-regulation of vascular endothelial growth factor (VEGF) (Krock and others, 2011; Nanka and others, 2006) which worked as a chemoattractant for NCCs (McLennan and others, 2010) thus promoting NCCs spreading. In either way, PHD inhibitors was able to restore NCCs defects in terms of *Sox9/10* and *Snail2* expression in embryos exposed to relatively high oxygen *ex ovo*. This raised a question as to whether PHD inhibitors exert a similar effect *in vivo* and, if so, whether they may be applicable to genetic diseases with NCC defects. To answer these questions we need to clarify whether PHD inhibitors give impact on all NCC derivatives or specific cell types, and whether they may cause any negative impact on development.

One of the HIF target genes relevant to NCCs is *Sox9* (Amarilio and others, 2007; Zhang and others, 2011). *Sox9* expression in the neural tube commits cells to undergo EMT, it then maintains cellular multipotency prior to lineage commitment (Cheung and Briscoe, 2003; McKeown and others, 2005). *Sox9* also promotes chondrogenesis by inducing cartilage-specific extracellular matrix genes such as collagen type II (Akiyama and others, 2002; Amarilio and others, 2007; Bell and others, 1997; Healy and others, 1999; Mori-Akiyama and others, 2003; Myllyharju and Schipani, 2010). Hence, the possible long-term impact that may arise by PHD inhibitors administration is enhanced chondrogenesis.

Here we investigate the effect of PHD inhibitors in chick embryos grown *in ovo* for up to 10 days, using three PHD inhibitors; dimethyloxalylglycine (DMOG) (Elvidge and others, 2006), a glycine-linked dipeptidyl-quinolone derivative IOX2 (Chowdhury and others, 2013) and an 8-hydroxyquinoline 7-substituent CCT1 (Thinnes and others, 2015). Following the application of the PHD inhibitors *in ovo*, increased expression of *Sox10* was observed as previously seen *ex ovo*, suggestive of increased NCCs. Vasculogenesis was also promoted temporality. The prominent effect was on development of the otic capsule, a cartilaginous structure encapsulating the inner ear formed by contribution of NCCs. Detailed analyses using micro-computed tomography

(μCT) showed deformed otic capsule and semicircular canals. The rather localised phenotype may be due to the unique dual origin of the skull, NCCs and mesoderm, as well as two distinct ossification mechanisms, endochondral and intramembranous ossification. The structures affected by PHD inhibitors were limited to NCCs-derived cartilaginous parts. The study shows the multiple effects and specificity of PHD inhibitors on developmental processes in an amniote model and supports the need for further study of their potential teratogenic effects in mammals.

Methods

Drug application in ovo, RNA *in situ* hybridisation, vessel labelling and Alcian blue staining

Fertilised chicken eggs were incubated for 30 hours at 38°C to obtain Hamburger and Hamilton stage (Hamburger and Hamilton, 1951) (HH) 5-6, opened with a window on the shell and injected with either DMSO (for control, Sigma), DMOG (Cayman), IOX2 (Sigma) and CCT1 (provided by Prof. C. Schofield, Oxford). Compounds were dissolved in DMSO at 0.5 M for DMOG and 0.1 M for IOX2 and CCT1, which were further diluted in Hanks' solution to 2 mM for DMOG and 0.2-0.8 mM for IOX2 and CCT1. Except for the evaluation experiment in Figure 1, compounds used for injection were DMOG 2 mM, IOX2 0.3 mM and CCT1 0.4 mM. DMSO was diluted at 1:250, the highest concentration used for drug dilution. Between 10-15 µl of the solution was injected in the space between the vitelline membrane and the blastoderm disk, using a mouth pipette. For administration at HH26, 25-30 µl of the solution was injected into the amniotic cavity. The window was sealed for continued incubation. Embryos were harvested and processed at required stages. RNA *in situ* hybridisation for *Sox10* (Cheng and others, 2000) was performed as previously described (Scully et al., 2016). Vessels were labelled by injecting with Lectin *Lens culinaris* Agglutinin (LCA) conjugated with rhodamine (Vector) by heart perfusion immediately before fixing with 4% paraformaldehyde. After removing the heart, embryos were flat-mounted. Alcian blue staining was performed as described (Behringer, 2014).

µCT scanning

Embryos were harvested 9 days post drug-application, washed in PBS and transferred to 4% paraformaldehyde in PBS overnight at 4°C. After washing with PBS, embryos were dehydrated with ethanol in a graded manner (50%, 70%, 90% and 100% twice) over a few hours, followed by staining with 1% Iodine in 100% ethanol overnight. Embryos were washed with 100% ethanol for 2 hours before scanning. CT images were obtained by scanning the embryos placed in a falcon tube with no solution. Scans were conducted on a Nikon XTH225ST CT scanner, using settings within the following ranges 70-80kV, 98-156uA, and 1000-1415 exposure times. Between 2000 and 3141 projections were taken, and most scans also averaged two frames per projection, resulting in scan lengths of up to 100 minutes. The variation in settings applied was a result of seeking optimal conditions for the best resulting tomograms in the time available, primarily to minimise noise in the resulting datasets. The projections were reconstructed using CT Pro 3D, resulting in TIF stacks of tomographic slices revealing

internal structures with voxel sizes of between 9.07 and 12.50 microns. VG Studio Max 3.0 (https://www.volumegraphics.com/) was utilised to reorient the tomogram slices into anatomical orientation, and to apply median filtering (size 3) to reduce noise in the slices (the latter applied only to the scans used for 3D reconstructions). Finally, the 3D visualisations of the otic capsule and inner ear were segmented out using a combination of semi-automated and manual segmentation tools in Avizo 8.1 software (https://www.fei.com/software/amira-avizo/). 3D models, Raw scan data and settings are available on https://data.bris.ac.uk/webshare/microCT_of_chick_embryonic_head/d317c0f5-5543-431b-aa6c-f08351b73883/. Further details and exact scan parameters in each case are provided in the readme.txt file accompanying the above datasets. SPSS software was used for ANOVA analyses.

Results

PHD inhibitors increase neural crest cells *in ovo*

To examine the effect of PHD inhibitors on NCCs in physiological conditions, each of DMOG, IOX2 and CCT1 as well as control DMSO, was applied *in ovo* at the head-fold stage (HH5-6, embryonic day (E) 1.5). The optimum concentration for IOX2 and CCT1 was empirically established by assessing *Sox10* expression at HH12 showing intense *Sox10* staining in NCCs compared to DMSO-treated embryos (Fig. 1), and was used throughout this study (see Methods). We found stronger staining of *Sox10* in NCCs at all cranial levels, similar to what was observed with DMOG in our previous *ex ovo* studies (Scully and others, 2016). The phenotype is likely due to augmented EMT that increased the number of neural crest cells as suggested previously (Scully and others, 2016). It is also possible that individual cells expressed *Sox10* more strongly as a regulator of hypoxic response (Li and others, 2013).

PHD inhibitors promote vasculogenesis

One of the major hypoxic responses in adults is angiogenesis that increases the local oxygen supply. In mouse embryos, HIF1α is required for proper vasculature development (Iyer and others, 1998; Ryan and others, 1998). It is also known in chick embryos that hypoxia upregulates the expression of VEGF and subsequent vasculogenesis (Nanka and others, 2006). In agreement with these, application of PHD inhibitors at HH5-6 promoted vasculogenesis as an immediate effect (Fig. 2). In normal embryogenesis, a pair of longitudinal dorsal aortae develop by HH12, which later fuse at the midline by a balance of signals including VEGF-A, forming the descending aorta (Garriock and others, 2010). It was noticed that PHD inhibitor-treated embryos tend to show fused dorsal aortae by HH12 at the level of the vitelline veins, the inflow tract to the heart (Fig. 2A-D), suggesting advanced vascular development. The width of the dorsal aortae tended to be greater in drug-treated embryos, however due to the natural variability along the vessels, it was not possible to quantify the width accurately. At HH13, the cephalic plexus begins to develop in the mesencephalon (Coffin and Poole, 1988) and drug-treated embryos showed higher density of the network (Fig. 2E-H). At HH25, however, the vessels and capillaries have developed in a dense manner in both control and drug-treated embryos and there was no apparent difference between them (Fig. 3A-J). Because of the recovered vessel phenotype in cephalic and pharyngeal regions at HH25, it appeared that the transiently increased vasculogenesis was not consolidated as a phenotype at a later stage, probably due to

the rapid and dynamic development of vasculogenesis by various regulatory mechanisms, including HIF as well as others (Ferguson and others, 2005).

In contrast to cephalic and pharyngeal levels, the trunk level between upper and lower limbs is yet to be formed when the drugs were applied at HH5-6. It was noted that, three days after the drug application (HH25), the developing trunk showed advanced vasculatures in embryos treated with PHD inhibitors (Fig. 3K-O), suggesting that the drug remained effective at least 3 days after the application and had worked on newly emerging structures.

PHD inhibitors promote chondrogenesis of the otic capsule

Next, the effect of PHD inhibitors on chondrogenesis was examined by Alcian blue staining (Fig. 4). The otic capsule arises as a cartilaginous structure located in the posterior-lateral aspect of the cranium, which eventually ossifies and provides protection to the inner ear located within it. The origin of the otic capsule is contentious (Gross and Hanken, 2008) but it is likely a mix of both NCCs (Le Lievre, 1978; Noden, 1983; O'Gorman, 2005) and head mesoderm (Couly and others, 1992). The inner ear, on the other hand, originates from the otic vesicle, which separates as the otocyst from the otic placode at HH18 (E3) (Chang and others, 2002). Small pouches start to develop at HH23-27 (E4-E5), which rapidly develop to loops at HH28-31 (E6-E7), thus forming superior (anterior), posterior and horizontal (lateral) semicircular canals (Bissonnette and Fekete, 1996; Chang and others, 1999). Such formed membranous labyrinth serves as a template of the inner ear when chondrogenesis occurs in the surrounding mesenchyme to form the otic capsule (Fig. 4A-F) (Chang and others, 1999; Chang and others, 2002; McPhee and Van de Water, 1986).

At HH30, embryos treated with PHD inhibitors at HH5-6 exhibited the structure of the otic capsule to be more complex compared to controls, resembling that of later stages (Fig. 4A-I). Three outpouches, the primordia of three semicircular canal aspects of the capsule, showed more advanced morphology, suggesting that PHD inhibitors caused accelerated development of the otic capsule. As HH30 was 5 days after the application of PHD inhibitors, it was not certain as to whether the drugs remained active and directly worked on chondrogenesis. To test the direct effect of the drugs, they were applied to embryos at HH26, immediately before the onset of chondrogenesis. Embryos treated with PHD inhibitors at HH26 showed advanced complexity of the otic capsule compared to the control (Fig. 4J-M), similarly to the ones with earlier injections, supporting the possibility that the drugs injected at HH5-6 have remained in the embryo and directly contributed to the advanced development of the otic capsule. The sustaining effect of the drug was in agreement with the result of promoted

vasculogenesis in the late-developing trunk region at HH25 suggesting the stability and bioactivity of the drugs *in vivo* at HH25.

The structure of the otic capsule is altered by PHD inhibitors

Due to the deep location and complex three-dimensional structure of the otic capsule, X-ray µCT scans were employed next. On HH36 (E10) chick embryos, it enabled image segmentation and 3D visualisation of the *in situ* structure of the otic capsule within the skull (Fig. 5). The application of DMOG was shown to significantly decrease the maximum width of the otic capsule (Fig. 5J) as well as the height (Fig. 5C,D). Changes to the dimensions of the otic capsule have distorted the inner ear lying within it (Fig. 6). The height of the anterior semicircular canal was reduced significantly in DMOG-treated embryos (Fig. 6K). The effect of CCT1 on the dimensions of the otic capsule and anterior semicircular canal also looked prominent but statistically not quite significant (see the legend of Figs. 5,6). Nonetheless, both CCT1 and IOX2 caused noticeably thinner semicircular canals compared to DMSO controls (Fig. 6E-H) presumably by compression by the surrounding otic capsule. Thus, although advanced development of the otic capsule was seen at E7 (Fig. 4), semicircular canals at HH36 were deformed and small in size. This is likely due to the nature of chondrogenesis, where matrix deposition would limit further morphogenesis. Based on these results, it was suggested that PHD inhibitors caused accelerated and premature chondrogenesis of the otic capsule.

As development of the otic capsule is under the influence of BMP signals emanating from the otic vesicle (Chang and others, 2002), the expression of *Msx1*, a target gene for BMP signals (Suzuki and others, 1997), was examined in embryos treated PHD inhibitors. There were not obvious changes observed for *Msx1* expression (Supplementary Fig. 1), ruling out a possible involvement of BMP signals in premature chondrogenesis caused by PHD inhibitors.

The formation of the palate and other cartilaginous structures are not affected by PHD inhibitors

Given the impact of PHD inhibitors on development of the otic capsule, we further examined development of other skeletal structures in the cranium. In contrast to the effect on the otic capsule, administration of PHD inhibitors at HH5-6 did not show apparent changes in the nasal capsule, Meckel's cartilage or tongue cartilages that had developed by HH34-36 (E8-10) (Supplementary Fig. 2). X-ray µCT analyses revealed that the maxilla and mandible as well as the structure of the eye were largely normal in all drug-treated embryos (Fig. 7A-D). We analysed in detail the palate, which

arises from NCCs and has a slit called the internal naris, similar to mammalian cleft palate; however, no significant change was found in drug-treated embryos (Fig. 7E,F).

Thus, the impact PHD inhibitors have in embryos is most prominent in the formation of the otic capsule, with transiently accelerated vasculogenesis.

Discussion

Why is the long-term effect of PHD inhibitors localised to the otic capsule?

In contrast to the increased expression of *Sox10* in NCCs at all cranial levels at HH12 (E2) (Fig. 1), the effect of PHD inhibitors at HH30-36 (Fig. 4-7) was rather localised to the otic capsule. The reason for this may lie in the multiple roles of PHD inhibitors on development of NCCs and dual origin and ossification processes of the skull. At the pre-migratory stage, PHD inhibitors likely increase EMT of NCCs, which was revealed by increased expression of *Snail2* (Scully and others, 2016). This might not be sufficient to cause drastic phenotypes in all NCC-derivatives, as further development of NCCs may compensate the increase during the proliferation, migration and differentiation processes, through interaction with the local environment and other NCCs (Carmona-Fontaine and others, 2008; Kulesa and Fraser, 2000; Noden, 1975; Noden and Trainor, 2005; Smith and others, 1997; Trainor and others, 2002). In order for PHD inhibitors to exert a structural effect, as well as promoting EMT of NCCs, it must also bias the cell fate of NCCs to that of a chondrocytic one and/or directly promote chondrogenesis itself.

NCCs undergo cell fate commitment over a long time span during development. They have some levels of lineage restriction in the neural tube prior to emigration (Krispin and others, 2010; Schilling and Kimmel, 1994) while maintaining plasticity (Noden, 1986; Trainor and others, 2003). A direct target of HIF, *Sox9/10*, may have helped maintain NCCs in an undifferentiated state (Dravis and others, 2015; McKeown and others, 2005) and promote chondrogenesis at later stages (Amarilio and others, 2007; Healy and others, 1999; Mori-Akiyama and others, 2003). The result of late-stage administration of PHD inhibitors at HH26 in regards to the otic capsule phenotype was similar to the one caused by HH5-6 administration (Fig. 3), suggesting that PHD inhibitors are stable for at least 3 days and able to promote chondrogenesis as well as increasing NCCs. The promoted vasculature in the trunk region seen at HH25 further reinforced the stability of the drug. Thus, multiple functions of PHD inhibitors at different stages of NCCs development are the key to cause the embryonic phenotype.

Facial and cranial vault structures arise from NCCs and paraxial mesoderm, and many ossify either endochondrally or intramembranously, while others remain as cartilage (Abzhanov and others, 2007; Couly and others, 1993; Noden and Trainor, 2005; Percival and Richtsmeier, 2013). Thus, only a limited number of bony structures, including the otic capsule, are formed with NCCs *and* undergo chondrogenesis for endochondral ossification. For example, the palate is a derivative of NCCs and

undergoes intramembranous ossification, thus PHD inhibitors would not work on the differentiation step, which may be the explanation why it was not affected by PHD inhibitors as seen in Figure 7.

Other structures that arise from NCCs and undergo chondrogenesis include the sclera, nasal capsule, Meckel's cartilage, columera, quadrate and hyoid, although some are contentious (Bellairs and Osmond, 2005; Le Douarin and Kalcheim, 1999). However, we did not find obvious effect of PHD inhibitors on nasal capsule, sclera and Meckel's cartilage at HH34-36 (supplementary Figure 2). With regard to the sclera and nasal capsule, this may be because of the fact that these structures persist as cartilage until postnatal or adulthood, which is different from most embryonic cartilage elements that are eventually replaced by bones relatively quickly. Interestingly, the chondrogenic differentiation process takes much longer in the sclera compared to that of limb, that allows sclera to adapt to the rapid growth of the eye while providing physical rigidity (Thompson and others, 2010). A similar rule may apply to the nasal capsule that changes the shape dramatically after pneumatisation (Smith and others, 2008). Hence, these cartilages in chick might be relatively refractory to factors that cause premature differentiation as was observed in the otic capsule. This is in contrast to the otic capsule, which grows very rapidly and develops morphological complexity in a short period of time at HH14-31 (Bissonnette and Fekete, 1996; Chang and others, 2002). Therefore, the timing and speed of organ development may be crucial for the susceptibility to the drugs, which is a common feature on critical period to teratogens.

It is to note that Waardenburg syndrome patients with *Sox10* mutations present enlarged vestibule and bilateral agenesis or hypoplasia of semicircular canals (Elmaleh-Berges and others, 2013; Sznajer and others, 2008). *Sox10* heterozygous mouse embryos are known to present reduced NCCs and migration problem (Southard-Smith and others, 1998). While the present study concerns embryos with increased *Sox10* expression, both studies highlight that the inner ear is sensitive to the *Sox10* expression level in the NCCs population.

Future perspective

Although physiological hypoxia is required for normal development of NCCs (Scully and others, 2016), this study has demonstrated that excess activation of the hypoxic pathway can have adverse effect on inner ear development. The adverse effect of PHD inhibitors on embryogenesis is important to note for the future clinical application of the drugs to childbearing women, given that PHD inhibitors are currently considered to be applicable to a wide range of ischemic diseases including renal anaemia (Brigandi and others, 2016; Maxwell and Eckardt, 2016; Provenzano and

others, 2016). In humans, the otocyst begins to form semicircular canals at the 5th week of gestation, that becomes surrounded by cartilaginous otic capsule by week 8, while early ossification begins after 20 weeks (Anson and Bast, 1958). The longer gestation period in human may suggest a lower chance of suffering from multiple effects of the drug, although it depends on its stability. It is yet to clarify whether or not a single effect of PHD inhibitors, e.g. an increase of NCCs alone, would have a long-term impact. It is also interesting to know whether the increase of NCCs by PHD inhibitors would have a rescuing effect on model animals with less NCCs, such as Tcof1-mutant model mouse for Treacher Collins syndrome (Dixon and others, 2006).

Acknowledgment

We thank Prof. C. Schofield for CCT1 compound, and Prof. P. Scotting for *Sox10* probe. This work was supported by the Anatomical Society. Authors do not have a conflict of interest to declare.

References

Abzhanov A, Rodda SJ, McMahon AP, Tabin CJ. 2007. Regulation of skeletogenic differentiation in cranial dermal bone. Development 134(17):3133-3144.

Ahlgren SC, Thakur V, Bronner-Fraser M. 2002. Sonic hedgehog rescues cranial neural crest from cell death induced by ethanol exposure. Proc Natl Acad Sci U S A 99(16):10476-10481.

Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrugghe B. 2002. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev 16(21):2813-2828.

Amarilio R, Viukov SV, Sharir A, Eshkar-Oren I, Johnson RS, Zelzer E. 2007. HIF1alpha regulation of Sox9 is necessary to maintain differentiation of hypoxic prechondrogenic cells during early skeletogenesis. Development 134(21):3917-3928.

Anson BJ, Bast TH. 1958. Development of the otic capsule of the human ear; illustrated in atlas series. Quarterly Bulletin of the Northwestern University Medical School 32:157–172.

Bajpai R, Chen DA, Rada-Iglesias A, Zhang J, Xiong Y, Helms J, Chang CP, Zhao Y, Swigut T, Wysocka J. 2010. CHD7 cooperates with PBAF to control multipotent neural crest formation. Nature 463(7283):958-962.

Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW, Sham MH, Koopman P, Tam PP, Cheah KS. 1997. SOX9 directly regulates the type-II collagen gene. Nat Genet 16(2):174-178.

Bellairs R, Osmond M. 2005. The atlas of chick development. 2nd ed. ed. Amsterdam ; London: Elsevier.

Bissonnette JP, Fekete DM. 1996. Standard atlas of the gross anatomy of the developing inner ear of the chicken. J Comp Neurol 368(4):620-630.

Brigandi RA, Johnson B, Oei C, Westerman M, Olbina G, de Zoysa J, Roger SD, Sahay M, Cross N, McMahon L, Guptha V, Smolyarchuk EA, Singh N, Russ SF, Kumar S, Investigators PHI. 2016. A Novel Hypoxia-Inducible Factor-Prolyl Hydroxylase Inhibitor (GSK1278863) for Anemia in CKD: A 28-Day, Phase 2A Randomized Trial. Am J Kidney Dis 67(6):861-871.

Bruick RK, McKnight SL. 2001. A conserved family of prolyl-4-hydroxylases that modify HIF. Science 294(5545):1337-1340.

Carmona-Fontaine C, Matthews HK, Kuriyama S, Moreno M, Dunn GA, Parsons M, Stern CD, Mayor R. 2008. Contact inhibition of locomotion in vivo controls neural crest directional migration. Nature 456(7224):957-961.

Cartwright MM, Smith SM. 1995. Increased cell death and reduced neural crest cell numbers in ethanol-exposed embryos: partial basis for the fetal alcohol syndrome phenotype. Alcohol Clin Exp Res 19(2):378-386.

Chang W, Nunes FD, De Jesus-Escobar JM, Harland R, Wu DK. 1999. Ectopic noggin blocks sensory and nonsensory organ morphogenesis in the chicken inner ear. Dev Biol 216(1):369-381.

Chang W, ten Dijke P, Wu DK. 2002. BMP pathways are involved in otic capsule formation and epithelial-mesenchymal signaling in the developing chicken inner ear. Dev Biol 251(2):380-394.

Cheng Y, Cheung M, Abu-Elmagd MM, Orme A, Scotting PJ. 2000. Chick sox10, a transcription factor expressed in both early neural crest cells and central nervous system. Brain Res Dev Brain Res 121(2):233-241.

Cheung M, Briscoe J. 2003. Neural crest development is regulated by the transcription factor Sox9. Development 130(23):5681-5693.

Chowdhury R, Candela-Lena JI, Chan MC, Greenald DJ, Yeoh KK, Tian YM, McDonough MA, Tumber A, Rose NR, Conejo-Garcia A, Demetriades M, Mathavan S, Kawamura A, Lee MK, van Eeden F, Pugh CW, Ratcliffe PJ,

Schofield CJ. 2013. Selective small molecule probes for the hypoxia inducible factor (HIF) prolyl hydroxylases. ACS Chem Biol 8(7):1488-1496.
Coffin JD, Poole TJ. 1988. Embryonic vascular development: immunohistochemical identification of the origin and subsequent morphogenesis of the major vessel primordia in quail embryos. Development 102(4):735-748.
Couly GF, Coltey PM, Le Douarin NM. 1992. The developmental fate of the cephalic mesoderm in quail-chick chimeras. Development 114(1):1-15.
Couly GF, Coltey PM, Le Douarin NM. 1993. The triple origin of skull in higher vertebrates: a study in quail-chick chimeras. Development 117(2):409-429.
Dixon J, Jones NC, Sandell LL, Jayasinghe SM, Crane J, Rey JP, Dixon MJ, Trainor PA. 2006. Tcof1/Treacle is required for neural crest cell formation and proliferation deficiencies that cause craniofacial abnormalities. Proc Natl Acad Sci U S A 103(36):13403-13408.
Dravis C, Spike BT, Harrell JC, Johns C, Trejo CL, Southard-Smith EM, Perou CM, Wahl GM. 2015. Sox10 Regulates Stem/Progenitor and Mesenchymal Cell States in Mammary Epithelial Cells. Cell Rep 12(12):2035-2048.
Elmaleh-Berges M, Baumann C, Noel-Petroff N, Sekkal A, Couloigner V, Devriendt K, Wilson M, Marlin S, Sebag G, Pingault V. 2013. Spectrum of temporal bone abnormalities in patients with Waardenburg syndrome and SOX10 mutations. AJNR Am J Neuroradiol 34(6):1257-1263.
Elvidge GP, Glenny L, Appelhoff RJ, Ratcliffe PJ, Ragoussis J, Gleadle JM. 2006. Concordant regulation of gene expression by hypoxia and 2-oxoglutarate-dependent dioxygenase inhibition: the role of HIF-1alpha, HIF-2alpha, and other pathways. J Biol Chem 281(22):15215-15226.
Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ. 2001. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 107(1):43-54.
Escot S, Blavet C, Faure E, Zaffran S, Duband JL, Fournier-Thibault C. 2016. Disruption of CXCR4 signaling in pharyngeal neural crest cells causes DiGeorge syndrome-like malformations. Development 143(4):582-588.
Ferguson JE, 3rd, Kelley RW, Patterson C. 2005. Mechanisms of endothelial differentiation in embryonic vasculogenesis. Arterioscler Thromb Vasc Biol 25(11):2246-2254.
Garriock RJ, Czeisler C, Ishii Y, Navetta AM, Mikawa T. 2010. An anteroposterior wave of vascular inhibitor downregulation signals aortae fusion along the embryonic midline axis. Development 137(21):3697-3706.
Gross JB, Hanken J. 2008. Review of fate-mapping studies of osteogenic cranial neural crest in vertebrates. Dev Biol 317(2):389-400.
Gupta N, Wish JB. 2017. Hypoxia-Inducible Factor Prolyl Hydroxylase Inhibitors: A Potential New Treatment for Anemia in Patients With CKD. Am J Kidney Dis 69(6):815-826.
Hamburger V, Hamilton HL. 1951. A series of normal stages in the development of the chick embryo. Journal of Morphology 88(1):49-92.
Healy C, Uwanogho D, Sharpe PT. 1999. Regulation and role of Sox9 in cartilage formation. Dev Dyn 215(1):69-78.
Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL. 1998. Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev 12(2):149-162.
Kiecker C. 2016. The chick embryo as a model for the effects of prenatal exposure to alcohol on craniofacial development. Dev Biol 415(2):314-325.

Krispin S, Nitzan E, Kassem Y, Kalcheim C. 2010. Evidence for a dynamic spatiotemporal fate map and early fate restrictions of premigratory avian neural crest. Development 137(4):585-595.
Krock BL, Skuli N, Simon MC. 2011. Hypoxia-induced angiogenesis: good and evil. Genes Cancer 2(12):1117-1133.
Kulesa PM, Fraser SE. 2000. In ovo time-lapse analysis of chick hindbrain neural crest cell migration shows cell interactions during migration to the branchial arches. Development 127(6):1161-1172.
Le Douarin N, Kalcheim C. 1999. The neural crest. 2nd ed. ed. Cambridge: Cambridge University Press.
Le Lievre CS. 1978. Participation of neural crest-derived cells in the genesis of the skull in birds. J Embryol Exp Morphol 47:17-37.
Li Q, Canosa S, Flynn K, Michaud M, Krauthammer M, Madri JA. 2013. Modeling the neurovascular niche: unbiased transcriptome analysis of the murine subventricular zone in response to hypoxic insult. PLoS One 8(10):e76265.
Markolovic S, Wilkins SE, Schofield CJ. 2015. Protein Hydroxylation Catalyzed by 2-Oxoglutarate-dependent Oxygenases. J Biol Chem 290(34):20712-20722.
Maxwell PH, Eckardt KU. 2016. HIF prolyl hydroxylase inhibitors for the treatment of renal anaemia and beyond. Nat Rev Nephrol 12(3):157-168.
McKeown SJ, Lee VM, Bronner-Fraser M, Newgreen DF, Farlie PG. 2005. Sox10 overexpression induces neural crest-like cells from all dorsoventral levels of the neural tube but inhibits differentiation. Dev Dyn 233(2):430-444.
McLennan R, Teddy JM, Kasemeier-Kulesa JC, Romine MH, Kulesa PM. 2010. Vascular endothelial growth factor (VEGF) regulates cranial neural crest migration in vivo. Dev Biol 339(1):114-125.
McMullin MF. 2010. HIF pathway mutations and erythrocytosis. Expert Rev Hematol 3(1):93-101.
McPhee JR, Van de Water TR. 1986. Epithelial-mesenchymal tissue interactions guiding otic capsule formation: the role of the otocyst. J Embryol Exp Morphol 97:1-24.
Mori-Akiyama Y, Akiyama H, Rowitch DH, de Crombrugghe B. 2003. Sox9 is required for determination of the chondrogenic cell lineage in the cranial neural crest. Proc Natl Acad Sci U S A 100(16):9360-9365.
Myllyharju J, Schipani E. 2010. Extracellular matrix genes as hypoxia-inducible targets. Cell Tissue Res 339(1):19-29.
Nanka O, Valasek P, Dvorakova M, Grim M. 2006. Experimental hypoxia and embryonic angiogenesis. Dev Dyn 235(3):723-733.
Noden DM. 1975. An analysis of migratory behavior of avian cephalic neural crest cells. Dev Biol 42(1):106-130.
Noden DM. 1983. The role of the neural crest in patterning of avian cranial skeletal, connective, and muscle tissues. Dev Biol 96(1):144-165.
Noden DM. 1986. Origins and patterning of craniofacial mesenchymal tissues. J Craniofac Genet Dev Biol Suppl 2:15-31.
Noden DM, Trainor PA. 2005. Relations and interactions between cranial mesoderm and neural crest populations. J Anat 207(5):575-601.
O'Gorman S. 2005. Second branchial arch lineages of the middle ear of wild-type and Hoxa2 mutant mice. Dev Dyn 234(1):124-131.
Percival CJ, Richtsmeier JT. 2013. Angiogenesis and intramembranous osteogenesis. Dev Dyn 242(8):909-922.
Provenzano R, Besarab A, Sun CH, Diamond SA, Durham JH, Cangiano JL, Aiello JR, Novak JE, Lee T, Leong R, Roberts BK, Saikali KG, Hemmerich S, Szczech LA, Yu KH, Neff TB. 2016. Oral Hypoxia-Inducible Factor Prolyl Hydroxylase Inhibitor Roxadustat (FG-4592) for the Treatment of Anemia in Patients with CKD. Clin J Am Soc Nephrol 11(6):982-991.

Rovasio RA, Battiato NL. 1995. Role of early migratory neural crest cells in developmental anomalies induced by ethanol. Int J Dev Biol 39(2):421-422.
Ryan HE, Lo J, Johnson RS. 1998. HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J 17(11):3005-3015.
Santagati F, Rijli FM. 2003. Cranial neural crest and the building of the vertebrate head. Nat Rev Neurosci 4(10):806-818.
Schilling TF, Kimmel CB. 1994. Segment and cell type lineage restrictions during pharyngeal arch development in the zebrafish embryo. Development 120(3):483-494.
Scully D, Keane E, Batt E, Karunakaran P, Higgins DF, Itasaki N. 2016. Hypoxia promotes production of neural crest cells in the embryonic head. Development 143(10):1742-1752.
Semenza GL. 2010. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 29(5):625-634.
Semenza GL. 2014. Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. Annu Rev Pathol 9:47-71.
Simoes-Costa M, Bronner ME. 2015. Establishing neural crest identity: a gene regulatory recipe. Development 142(2):242-257.
Smith A, Robinson V, Patel K, Wilkinson DG. 1997. The EphA4 and EphB1 receptor tyrosine kinases and ephrin-B2 ligand regulate targeted migration of branchial neural crest cells. Curr Biol 7(8):561-570.
Smith TD, Rossie JB, Docherty BA, Cooper GM, Bonar CJ, Silverio AL, Burrows AM. 2008. Fate of the nasal capsular cartilages in prenatal and perinatal tamarins (Saguinus geoffroyi) and extent of secondary pneumatization of maxillary and frontal sinuses. Anat Rec (Hoboken) 291(11):1397-1413.
Southard-Smith EM, Kos L, Pavan WJ. 1998. Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. Nat Genet 18(1):60-64.
Suzuki A, Ueno N, Hemmati-Brivanlou A. 1997. Xenopus msx1 mediates epidermal induction and neural inhibition by BMP4. Development 124(16):3037-3044.
Sznajer Y, Coldea C, Meire F, Delpierre I, Sekhara T, Touraine RL. 2008. A de novo SOX10 mutation causing severe type 4 Waardenburg syndrome without Hirschsprung disease. Am J Med Genet A 146A(8):1038-1041.
Tan TY, Kilpatrick N, Farlie PG. 2013. Developmental and genetic perspectives on Pierre Robin sequence. Am J Med Genet C Semin Med Genet 163C(4):295-305.
Thinnes CC, Tumber A, Yapp C, Scozzafava G, Yeh T, Chan MC, Tran TA, Hsu K, Tarhonskaya H, Walport LJ, Wilkins SE, Martinez ED, Muller S, Pugh CW, Ratcliffe PJ, Brennan PE, Kawamura A, Schofield CJ. 2015. Betti reaction enables efficient synthesis of 8-hydroxyquinoline inhibitors of 2-oxoglutarate oxygenases. Chem Commun (Camb).
Thompson H, Griffiths JS, Jeffery G, McGonnell IM. 2010. The retinal pigment epithelium of the eye regulates the development of scleral cartilage. Dev Biol 347(1):40-52.
Trainor PA. 2010. Craniofacial birth defects: The role of neural crest cells in the etiology and pathogenesis of Treacher Collins syndrome and the potential for prevention. Am J Med Genet A 152A(12):2984-2994.
Trainor PA, Melton KR, Manzanares M. 2003. Origins and plasticity of neural crest cells and their roles in jaw and craniofacial evolution. Int J Dev Biol 47(7-8):541-553.
Trainor PA, Sobieszczuk D, Wilkinson D, Krumlauf R. 2002. Signalling between the hindbrain and paraxial tissues dictates neural crest migration pathways. Development 129(2):433-442.
Yeh T-L, Leissing TM, Abboud MI, Thinnes CC, Atasoylu O, Holt-Martyn JP, Zhang D, Tumber A, Lippl K, Lohans CT, Leung IKH, Morcrette H, Clifton IJ, Claridge TDW, Kawamura A, Flashman E, Lu X, Ratcliffe PJ, Chowdhury R,

Pugh CW, Schofield CJ. 2017. Molecular and cellular mechanisms of HIF prolyl hydroxylase inhibitors in clinical trials. Chemical Science 8:7651-7668.
Zhang C, Yang F, Cornelia R, Tang W, Swisher S, Kim H. 2011. Hypoxia-inducible factor-1 is a positive regulator of Sox9 activity in femoral head osteonecrosis. Bone 48(3):507-513.

Figure legends

Fig. 1. Increase of neural crest cells by PHD inhibitors *in vivo*.

DMSO controls (A,F) shows all three streams are well established (white arrows). IOX2 (B-E) or CCT1 (G-J) treated embryos also show all migrations streams present. Black arrows show increased *Sox10*-positive cells. In C,E and J the incidence of sustained induction of NCC from the dorsal aspect of the neural tube is observed (arrowheads), as seen in our previous study (Scully et al., 2016). The stacked column chart (K-M) indicates how the embryos were affected, with an increase (black) in NCC, comparable to controls (white) or decrease in NCC and/or toxic to embryos (grey). The highest concentration of both IOX2 and CCT1 (0.8mM) does not necessarily further the increase (black), rather, it shows underdevelopment of embryos and toxicity (grey). For IOX2 (K, 0.3mM n=15, 0.4mM n=10, 0.8mM n=7), the optimum concentration is shown to be 0.3mM as the highest incidence of the increase of NCCs. 0.2mM concentration for IOX2 was omitted due to a small sample size (n=1). For CCT1 (L, 0.2mM n=3, 0.3mM n=10, 0.4mM n=17, 0.8mM n=5), the optimum concentration was concluded to be 0.4mM. DMOG was tested only at the concentration of 2mM (M, n=11). Scale bar in J, 200 µm.

Fig. 2. Vasculature development one day after PHD inhibitor application.

Embryos were treated with DMSO, DMOG, IOX2 and CCT1 at HH5-6, injected with LCA and fixed at HH12 or 13. The heart was removed at vitelline veins (the cut end is indicated by asterisks) and arteries proximal to the heart. Embryos were either whole mounted (A-D) or cut into sagittal halves and flat-mounted (E-H). Bright field and dark field are shown.

(A-D) HH12, dorsal view. Right and left dorsal aortae (DA) have developed and a sign of fusion (arrow) is seen at the level of vitelline veins (DMSO, n=0/4; DMOG, n=4/10; IOX2, n=3/6; CCT1, n=1/3). Scale bar; 200 µm.

(E-H) HH13, lateral view. The mesencephalon (Mes) and diencephalon (Di) have developed cephalic plexus, which is more advanced in embryos treated with DMOG (n=3), IOX2 (n=7) or CCT1 (n=3) compared to the ones with DMSO (n=5). Scale bar; 200 µm.

Fig. 3. Vasculature development three days after PHD inhibitor application.

Embryos were treated with DMSO, DMOG, IOX2 and CCT1 at HH5-6, injected with LCA and fixed at HH25. The cephalic, pharyngeal and trunk regions are shown as indicated. (A,F,K) show lower magnification of DMSO-treated embryos, with rectangles indicating the area shown in (B,G,L). In all embryos, vessels and capillaries have developed in a similar degree at the cephalic and pharyngeal regions (DMSO, n=10; DMOG, n=12; IOX2, n=3; CCT1, n=4). Whereas in the trunk region, more vessels are seen in segments between intersomitic vessels in drug-treated embryos compared to DMSO-treated embryos (DMOG, n=12/12; IOX2, n=3/3; CCT1, n=3/4). Tel, telencephalon; Di, diencephalon; Mes, mesencephalon; ba1 and ba2, branchial arch 1 and 2. Arrowheads show three segments divided by intersomitic vessels. Scale bars; 500 μm.

Fig. 4. Advanced development of the otic capsule in PHD inhibitor-treated embryos.

(A-F) A chronological series of Alcian blue-stained embryos at HH29-33 depicting the increasing complexity of the otic capsule in DMSO treated embryos, with schematic drawings below. The rectangle in (A) is the area magnified in (B). During the stages HH29-33 (E6-8), the dorsal aspect of the otic capsule becomes defined as the superior outpouch develops, whereas in the anterior aspect the horizontal outpouch elongates and posteriorly the posterior outpouch develops the curvature. a, anterior; d, dorsal; l, lateral; post, posterior outpouch; sup, superior outpouch; hor, horizontal outpouch; cd, cochlear duct.

(G-I) Embryos at HH30, treated with PHD inhibitors as indicated at HH5-6. Two examples are shown for each treatment. Well-defined otic capsules resemble HH31-32; in particular in horizontal and posterior outpouches. Advanced outpouches compared to DMSO were seen in; DMOG, n=8/8; IOX2, n=9/9; CCT1, n=4/4.

(J-M) Embryos at stage HH31, treated with PHD inhibitors as indicated at HH26. Two examples are shown for each treatment. Outpouches are more well defined in drug-treated embryos (K-M) compared to the control (J). Advanced outpouches compared to DMSO were seen in; DMOG, n=5/5; IOX2, n=9/9; CCT1, n=7/7.

Fig. 5. Deformed otic capsules after PHD inhibitor treatment

(A-H) Three-Dimensional reconstructions of the otic capsule (blue) and inner ear (green) of 10-day-old chick embryos (HH36) after DMSO or PHD inhibitor treatment,

posterior (A,C,E,G,I) and left posterior oblique (B,D,F,H) views. The DMSO control (A,B) shows a tall and wide otic capsule, with a large foramen magnum in the centre. In DMOG-treated embryos (C,D) the height of the otic capsule is reduced. This has in turn lead to the distortion of the inner ear within the otic capsule, as shown in Fig. 5. The height of the otic capsule in IOX2 (E,F) and CCT1 (G,H) -treated embryos does not appear affected (E-H). The maximum width of the otic capsule (indicated by white arrow in I) was quantified in 6 embryos from each group, resulting in graph J. The width of the otic capsule was relatively reduced in DMOG or CCT1 treated embryos. A statistically significant difference was shown between DMOG and IOX2 (ANOVA, P=0.027). The bars show the mean of 6 embryos and the error bars show standard deviation.

Fig. 6. Deformed semicircular canals after PHD inhibitor treatment

Three-Dimensional reconstructions of the inner ear of 10-day-old chick embryos (HH36) after DMSO or PHD inhibitor treatment, posterior (A,C,E,G) and left-posterior oblique views (B,D,F,H). Semicircular canals (a, anterior; l, lateral; p, posterior) and cochlear duct (c) are indicated. In DMOG-treated embryo, the height of the anterior semicircular canal is noticeably reduced (C,D, double arrowhead). In IOX2 or CCT1-treated embryos, the semicircular canals are strikingly thinner and discontinuous (E-H, arrowheads). The cochlear duct is kept intact in all cases. (I,J) Left lateral views showing the orientation and measurement for the distance between the ampulla and the tallest aspect of the anterior semicircular canal (measured in 3D) used for (K). Graph (K) shows the result of 6 embryos (12 sides) for each of the four groups. The difference in height was statically significantly different between DMSO and DMOG (P=0.005) and DMOG and IOX2 (P=0.011) in ANOVA analyses. CCT1 also appeared affected but statistically not quite significant (P<0.1). The bars show the mean of 12 anterior semicircular canals from 6 embryos and the error bars show standard deviation.

Fig. 7. Coronal CT image of the maxilla in 10-day-old chick embryos after application of PHD inhibitors

(A-D) shows coronal section including the maxilla and mandible, including the internal naris, a slit between bilateral palatine processes (see arrows in E for the position). Embryos treated with PHD inhibitors show similar size of internal naris (B-D) compare to DMSO control (A). (E) shows a magnified image of the maxilla with internal naris

(arrow). The size of the slit was quantified, and graph (F) was produced from data of 6 embryos of each group. (F) shows that the size of internal naris was not altered through application of PHD inhibitors, assessed by ANOVA. The error bars show standard deviation.