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Expression of Prolyl hydroxylases 2 and 3 in chick embryos

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Abstract

Hypoxic cellular response is crucial for normal development as well as in pathological conditions in order to tolerate low oxygen. The response is mediated by Hypoxia Inducible Factors (HIFs), where the α-subunit of HIF is stabilised and able to function only in low oxygen. Prolyl hydroxyrases (PHDs) are oxygen dependent dioxygenase enzymes that hydroxyrate HIF-α leading to HIF degradation. Thus PHDs function as an oxygen sensor for the function of HIFs. Here we describe the mRNA expression pattern of PHDs in chick embryos. Up to embryonic day 2, PHDs are weak without specific localisation, whereas from day 3 localised expression was observed in the eye, branchial arches and dermomyotome. Later in the limb development PHDs were expressed in the perichondral mesenchyme, excluded from the developing limb cartilages.
Introduction

Tolerance to hypoxia is essential in embryonic development (Dunwoodie, 2009) as well as in ischemic conditions in adults (Semenza, 2014). Hypoxic cellular response is mostly exerted via the activity of Hypoxia Inducible Factor (HIF), a heterodimeric complex composed of an oxygen-sensitive HIF-α and a constitutively expressed HIF-β subunit (Wang et al., 1995). As a transcription factor, HIF targets over 100 genes to counteract the reduction in oxygen levels at the cellular, local tissue and systemic level (Liu et al., 2012). The main role of HIFs includes stimulating angiogenesis with increased VEGF transcription, promoting erythropoiesis, enhancing glucose uptake and glycolysis and engaging programmed cell death for adaptation (Biju et al., 2004; Haase, 2013; Lu et al., 2002; Shweiki et al., 1992). Hypoxia has also been linked to disease processes such as cardiac abnormalities (Bishop and Ratcliffe, 2015) and tumour metastasis (Semenza, 2013).

The primary means of controlling HIF activity in response to oxygen concentration is oxygen-dependent hydroxylation of specific proline residues of the α-subunit, which promotes an association with the von Hippel-Lindau ubiquitin E3 ligase and subsequent destruction of HIF-α (Bruick and McKnight, 2001). HIF prolyl hydroxylation is catalysed by prolyl hydroxylase domain (PHD) enzymes 1-3, also known as Egln-2, -1 and -3 respectively, that are members of the iron and oxygen dependent, 2-oxoglutarate-dependent dioxygenase family (Kaelin and Ratcliffe, 2008). All three PHDs are similar at the C-terminus containing prolyl-4-hydroxylase-sequence, but PHD2 has a zinc finger at its N-terminus absent from the other PHDs (Place and Domann, 2013). PHDs act as oxygen sensors that keep HIF at low levels during normoxia. On the contrary, when oxygen is not available, PHDs do not hydroxylate HIF-α, thus allowing HIF-α proteins to be stabilised and function.
PHDs are expressed in different cell types and tissues, and contribute to the physiological regulation of HIF-α (Appelhoff et al., 2004; Berra et al., 2003; Lieb et al., 2002). PHDs are transcriptionally up-regulated by HIF-α and therefore involved in a feedback loop with HIF during prolonged hypoxia or rapid degradation upon re-oxygenation (Loor and Schumacker, 2008; Speer et al., 2013).

The importance of HIF-1α in embryogenesis was demonstrated by targeted deletion that caused a significant decrease in vascularisation and abnormal head fold formation manifest at 8.5-9.5 dpc (Iyer et al., 1998; Ryan et al., 1998). With regard to PHDs, PHD2 knock-outs in mouse leads to embryonic lethality at 12.5-14.5 dpc, whereas deletion of PHD1 or PHD3 does not affect viability (Takeda et al., 2006). The major cell differentiation processes during embryogenesis where the HIF pathway is likely involved are vasculogenesis and chondrogenesis. In addition, epithelial to mesenchymal transition (EMT) may also be initiated by HIF, as HIF directly regulates Snail1 expression (Imai et al., 2003; Luo et al., 2011).

The expression of HIF-1α and HIF-2α in embryos has been described in detail in various species (de Beaucourt and Coumailleau, 2007; Etchevers, 2003; Köblitz et al., 2015; Ota et al., 2007). However, description on PHD expression is largely limited to adult tissues and cell lines (Appelhoff et al., 2004; Lieb et al., 2002); the only description in embryos is in *Xenopus*, where PHDs are expressed in the brain, branchial arches, otic vesicles and pronephros (Han et al., 2012). PHD3 is also expressed in the heart and somites (Han et al., 2012). However, as aquatic animals *Xenopus* may have roles and regulatory mechanisms for the HIF pathway different from amnionotes during embryogenesis. Here we describe expression pattern of PHDs in chick embryos focusing on early developmental stages. It should be noted that, in
contrast to other vertebrates that have three PHD genes, chick embryos appear to have only two; PHD2 and PHD3.
Results and Discussion

Gene homology search on Ensembl (http://www.ensembl.org) showed that chicks have only two PHDs; PHD2 (EGLN1) and PHD3 (EGLN3) (Fig. 1). Chick PHD3 showed a similarity in both size and amino acid sequences (%) to those of other species, whereas chick PHD2 is smaller (316 amino acids) compared to PHD1 or PHD2 of other species (≥400 amino acids). Thus chicks appear to have unique sets of PHDs compared to other vertebrates. The avian species that are sequenced on Ensembl are Chicken, Turkey, Duck, Flycatcher and Zebra Finch. Among those, Chicken and Turkey have exclusively EGLN1 and 3, whereas Duck, Flycatcher and Zebra Finch have EGLN1, EGLN3 and another gene called P4H-TM (prolyl 4-hydroxylase, transmembrane, which may also be called PHD4/EGLN4 (Koivunen et al., 2007). PHD4/EGLN4 is found in most of vertebrate species such as Humans, Mouse, Xenopus and Zebrafish. None of the avian species mentioned above have PHD1 (EGLN2) in the Ensembl search. Therefore, the lack of PHD1 appears to be common in avian species.

Chick embryos at Hamburger and Hamilton (Hamburger and Hamilton, 1951) stages 8-13 showed faint and broad staining of both PHD2 and PHD3 (Fig. 2A-F). To note, at this stage HIF1-α is expressed in embryo proper, in a broad manner similarly to PHDs, whereas HIF2-α is in extraembryonic tissues (Ota et al., 2007).

To examine whether the expression of PHD2/3 changes in response to availability of oxygen or by stabilisation of HIF, the expression was compared between in ovo and ex ovo cultured embryos, along with the ones cultured ex ovo with dimethyloxalyl glycine (DMOG) (Fig. 1G-L). The difference of staining between three conditions was marginal in both PHD2 and PHD3, although, there was a trend that ex ovo cultured embryos showed slightly weaker staining of PHD3 compared to
that of in ovo (n=4/6; no apparent difference for PHD2, n=0/6) whereas DMOG treatment caused stronger staining compared to control ex ovo cultures (n=4/6 for PHD2, n=5/6 for PHD3).

At stage 19, localised staining of PHD2 was seen in the telencephalon, dorsal mesencephalon, the eye and optic stalk, the maxillary process and 1\textsuperscript{st} and 2\textsuperscript{nd} pharyngeal arches (Fig. 3A,B). A periodic expression in the somite was also observed (Fig. 3A). Transverse sections of the trunk showed PHD2 expression in the apical side of epithelialized somites (Fig. 3G-I) and in the dermomyotome in more differentiated somites (Fig. 3F). The expression of PHD3 was similar to PHD2 in the cranial region at this stage, however, the expression in the somites was not prominent (Fig. 3C-E).

By stage 21, localised expression of PHD2 in the eye, the maxillary process and 1\textsuperscript{st} and 2\textsuperscript{nd} pharyngeal arches had become prominent (Fig. 4A). It has also become noticeable that the heart is relatively weak in staining. This is in agreement with the localised expression of HIF-1\alpha in the developing heart (Nanka et al., 2006).

The expression pattern of PHD2 in somites had also become distinct by stage 21 (Fig 4B,E,F). Histological sections showed that the expression is localised to the ventral side of the dermomyotome (Fig. 4E) and further localised to the medial part in more differentiated (anterior) levels (Fig. 4F). In contrast, PHD3 did not show such specific expression in somites, while the expression in the eyes and branchial arches are maintained (Fig. 3C,D,G).

The developing limb buds showed a broad expression of both PHD2 and PHD3 (Fig. 4H-K). At stage 27, when the digital cartilages have become prominent, both PHD2 and PHD3 were expressed in the inter-digital mesenchyme, particularly at the edges of the cartilage, with clear exclusion from the cartilage (Fig. 5A-G). This expression was also observed at stage 32 when the digits are elongated (Fig. 5H,I).
There was no obvious difference between the expression of PHD2 and PHD3 in the developing limb. Histological section showed the PHD-expression is in the limb mesenchyme underneath the surface ectoderm (Fig. 5J).
Methods and methods

Chick embryos were purchased from local farm and incubated at 38°C in humidified incubator. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). In situ hybridisation was performed as described previously (Acloque et al., 2008). Cartilage staining was performed as described (Bronner-Fraser, 2008). In aligning the protein structure of PHDs, mouse PHD structures were based on; PHD1, AF453879; PHD2, NM_053207; PHD3, NM_028133. Chick PHD2 and PHD3 were based on XM_001231253 and XM_421233, respectively. Xenopus PHD structures were based on; PHD1 NM_001167742; PHD2, NM_001093091; PHD3, NM_001127012. Chick PHD2 and PHD3 probes, antisense and sense, were synthesized using Chick EST clone 400i24 (880 bp) and 680g23 (490 bp), respectively. The PHD2 clone was chosen based on the matching with XM_001231253, including a part of the coding region and 285 base pair of 3’UTR. The PHD3 clone was chosen based on the matching to both XM_421233 and XM_004941757, that are identical at 1-229 amino acids whereas the C-terminal 12 and 20 amino acids, respectively, are different.

Ex ovo culture was performed on agar plate as described (Chapman et al., 2001) with either 0.1% DMSO or 2mM DMOG (Cayman Chemical) dissolved in the agar. The amount of DMSO was the same for both ex ovo control and DMOG cultures. Embryos were taken out of the shell at HH stages 5-7, cultured in ambient air on the agar plate and further incubated for 24 hours before fixing.
REFERENCES


**Figure legends**
**Fig. 1.** Schematic drawing of mouse chick and Xenopus PHD proteins. The shaded region shows prolyl-4-hydroxylase alpha subunit containing (P4Hc) sequence. Numbers indicate amino acid positions.

**Fig. 2.** *PHD2* and *PHD3* expression in chick embryos at stages 8-13.

(A-F) HH stages 8 (A, D) and 13 (B,C,E,F) showing PHD2 (A,B) and PHD3 (D,E) expression with antisense probe, and negative controls with sense probes (C,F). There is no distinct localisation of expression at these stages. (B) and (C) are stained in parallel hence comparable, so are (E) and (F). Scale bar, 500 µm.

(G-L) HH stage 11-12 embryos incubated *in ovo* (G,J), *ex ovo* with control DMSO (H,K) or *ex ovo* with DMOG (I,L), stained with PHD2 (G-I) or PHD3 (J-L). DMOG treated embryos show slightly stronger staining compared to control embryos cultured *ex ovo* with DMSO (H,I; K,L). Embryo cultured *ex ovo* with DMSO shows a slightly weaker staining of PHD3 compared to *in ovo* cultured embryo (J,K).

**Fig. 3.** *PHD2* and *PHD3* expression in chick embryos at stage 19.

(A-E) HH stage 19 embryos stained with PHD2 (A,B) and PHD3 (C-E). Localised staining of PHD2 is seen in the telencephalon, dorsal mesencephalon, the eye and optic stalk (arrowhead), the maxillary process and 1st and 2nd pharyngeal arches (arrows), as well as in somites (small arrowheads). (B) is a coronal section crossing the midbrain and pharyngeal arches. ph1; pharyngeal arch 1. The expression pattern of PHD3 (C) is similar to PHD2 except the absence in somites. (D) and (E) were stained in parallel with either antisense (D) or sense (E) probes of PHD3. Scale bars in (B), 200 µm; (C), 100 µm.
(F-I) Transverse sections of the trunk showing PHD2 expression in the dermomyotome (F) and in the apical side of epithelialized somites (G-J). Scale bar, 100 µm.

**Fig. 4.** PHD2 and PHD3 expression in chick embryos at stages 19-21.

(A-D) Whole mount embryos stained with PHD2 (A,B) and PHD3 (C,D). Both PHD2 and PHD3 show localised expression in the eye, the maxillary process and 1st and 2nd pharyngeal arches (A,C), however, expressions in somites are seen only for PHD2 (B) and not PHD3 (D). Scale bar, 500 µm.

(E-G) Histological sections at trunk levels showing PHD2 (E,F) and PHD3 (G) expression. (F,G) are more anterior than (E). PHD2 expression is localised to the ventral side of the dermomyotome (E) or the medial part of it in more anterior levels (F). PHD3 does not show prominent expression in somites (G). Scale bar, 200 µm.

(H-K) Whole mount trunk region of stage 19 embryos at the forelimb level stained with PHD2 (H) and PHD3 (J). (I,K) are with sense probes of each. Scale bar, 200 µm.

**Fig. 5.** PHD2 and PHD3 expression in chick limb buds.

(A-G) Whole mount (A,C) and section (B,D) of PHD2 (A,B) and PHD3 (C,D) expression at stage 27, showing expression in the inter-digital mesenchyme excluding from the cartilage. Alcian blue staining is shown in (E) for comparison. (F,G) are staining with antisense and sense probes of PHD2, respectively. Scale bars, 500 µm.

(H-J) Whole mount (H,I) and section (J) of PHD2 (H) and PHD3 (I,J) expression at stage 32. Digital cartilages are negative for both PHD2 and PHD3. (J) shows that PHD3 expression is excluded from the surface ectoderm (bracket). There was no
obvious difference between the expression of PHD2 and PHD3 in the developing limb. Scale bars in (H), 500 µm; (J), 25 µm.
Mouse PHD1/EGLN2 (419 aa)

Mouse PHD2/EGLN1 (400 aa)

Mouse PHD3/EGLN3 (239 aa)

Chick PHD2/EGLN1 (316 aa)

Chick PHD3/EGLN3 (241 aa)

Xenopus PHD1/EGLN2 (454 aa)

Xenopus PHD2/EGLN1 (408 aa)

Xenopus PHD3/EGLN3 (252 aa)
Figure 2
Figure 5