The role of nitric oxide during embryonic epidermis development of *Xenopus laevis*

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**ABSTRACT**

Nitric oxide (NO) is a potent radical molecule that participates in various biological processes such as vasodilation, cell proliferation, immune response and neurotransmission. NO mainly activates soluble guanylate cyclase, leading to cGMP production and activation of protein kinase G and its downstream targets. Here we report the essential role of NO during embryonic epidermis development. *Xenopus* embryonic epidermis has become a useful model reflecting human epithelial tissue composition. The developing epidermis of *Xenopus laevis* is formed from specialized ionocytes, multi-ciliated, goblet and small secretory cells. We found that NO is mainly produced in multi-ciliated cells and ionocytes. Production of NO during early developmental stages is required for formation of multi-ciliated cells, ionocytes and small secretory cells by regulation of epidermal-specific gene expression. The data from this research indicate a novel role of NO during development, which supports recent findings of NO production in human mucociliary and epithelium development.

**KEY WORDS:** Development, Epidermis, Nitric oxide, *Xenopus laevis*, Mucociliary epithelium

**INTRODUCTION**

*Xenopus laevis* has been recently introduced as a great model for mucociliary and epidermal research (Dubaissi and Papalopulu, 2011; Noiret et al., 2016). *Xenopus* epidermis show similarity in cellular composition with human mucus and mucociliary epithelium and also share similar genetically encoded defects especially for abnormalities during respiratory tract development (Dubaissi and Papalopulu, 2011; Walentek et al., 2014, 2015). Matured epidermis of *Xenopus* tadpoles is a bilayered epithelium (like mammalian embryonic epidermis) with the outer layer composed of specialized cell types (goblet, ion secreting, small secretory and multi-ciliated cells) and the inner layer of undifferentiated cells (Chang and Hemmati-Brivanlou, 1998).

The first differentiation of epidermal precursors to precursor cells occurs during gastrulation in the inner layer (Billett and Gould, 1971). The precursors of multi-ciliated cells (MCCs) are specified by Notch-mediated lateral inhibition. Cells destined to MCC fate, express Notch ligand Delta and activate the Notch pathway in neighbouring cells. Activated Notch leads to expression of genes, which prevent transformation of these neighbouring cells to ciliated fate (Wettstein et al., 1997; Deblandre et al., 1999). Precursors of ciliated cells migrate by radial intercalation during neurulation from the inner to the outer layer in a ‘salt and pepper’ pattern (Deblandre et al., 1999; Marnelllos et al., 2000; Stubbs et al., 2006). Subsequently, these cells undergo ciliogenesis at stages 22-28 (Nieuwkoop and Faber, 1944) and become mature MCCs with coordinated polarization at stage 30 (Mitchell et al., 2007). MCCs are probably responsible for the transport of extracellular fluids along the embryonic skin by cilia beating in a single polarized direction. Complex molecular regulation of ciliated epithelia development is poorly understood and its importance was shown in studies of defective ciliogenesis associated with the broad spectrum of human ciliopathies (Mall, 2008; Marshall and Kintner, 2008).

In addition to MCCs, three other cellular types can be distinguished in the outer layer of matured embryonic epidermis. Ionocytes [sometimes called intercalating non-ciliated cells (INCs)] are specified by Notch signalling during gastrulation and intercalate into the outer layer of the epidermis like MCCs, and form a scattered distribution often near or in contact with MCCs. Ionocytes are divided into two subgroups (alpha and beta subtypes) based on their differing expression levels of foxi1e, ca12 and atp6v1a. Ionocytes are responsible for pH regulation by the activity of their membrane pumps and channels and they also play a crucial role in the development of a functional mucociliary epithelium. Depletion of ionocytes leads to defects in MCCs (Stubbs et al., 2006; Dubaissi and Papalopulu, 2011; Quigley et al., 2011). Recently, another cell type within the outer layer of the epidermis, the small secretory cells (SSCs), has been characterized. SSCs contain apically localized large vesicles containing serotonin, which is a monoamine derived from tryptophan modification. Development of SSCs is regulated by the activity of the transcription factor Foxa1. Expression of foxa1 first appears in the inner layer during early neurulation (Dubaissi et al., 2014). SSCs intercalate into the outer layer of the epidermis during stages 27-30, later than that of MCCs and ionocytes. Matured SSCs synthesize and secrete serotonin and additional substances on the surface of the embryo to provide it some anti-bacterial function. SSCs regulate motility and beating frequency of MCCs through serotonin receptor Htr3 at the later stages of embryonic development (Dubaissi et al., 2014; Walentek et al., 2014). Goblet cells are the fourth epithelial cell type. In contrast to the other cell types, goblet cells do not intercalate from the inner epithelial layer, but instead develop and form most of the outer layer. Goblet cells are known to secrete a lectin Xeel and probably have a protective function in the embryonic epidermis (Billett and Gould, 1971; Nagata et al., 2003).

It is well known that matured epidermis is susceptible to many chemical and physical factors. One of the main factors influencing skin is UV light, which causes production of reactive oxygen...
species (ROS) and reactive nitrogen species (RNS) within the epidermis (Peus et al., 1998; Fisher et al., 2002; Chang et al., 2003; Ichihashi et al., 2003). Although there is plenty of information about the negative effect of ROS and RNS on matured epidermis, almost nothing is known about their positive role during embryonic epidermis development. A very interesting molecule from the RNS group is nitric oxide (NO), which is a small signalling molecule involved in a wide range of biological processes (both physiological and pathological) like vasodilatation, neurotransmission and apoptosis (Palmer et al., 1987; Albina et al., 1993; West et al., 2002). It is generated by three nitric oxide synthases (NOS): constitutively active neuronal, nNOS/NOS1; endothelial, eNOS/NOS3; and inducible, iNOS/NOS2 (Bredt et al., 1991; Knowles and Moncada, 1994). NO activates a soluble isozyme of guanlyl cyclase (sGC) that induces the production of cGMP followed by the activation of protein kinase G (PRKG) regulating transcription and translation of downstream target genes (Arnold et al., 1977; Francis et al., 2010), and, alternatively, acts independently on the cGMP pathway (Cui et al., 2005; Oberprieler et al., 2007). NO production has been detected in the human skin (Wang et al., 1996; Shimizu et al., 1997; Chang et al., 2003), but its function is unclear.

NO is crucial during embryogenesis, especially during craniofacial development (Gouge et al., 1998; Jacox et al., 2014). NO was found to be highly produced in embryonic epidermis of various animal models (Brunelli et al., 2005; Alpert et al., 2007; Wildling and Kerschbaum, 2007; Jacox et al., 2014). Furthermore, its production and function were studied in the human pulmonary epithelium, ciliary motility and ciliary beating frequency (Jain et al., 1997; Jiao et al., 2010). Neuronal version of NOS was discovered in human cilia and airway epithelium localized at the proximal portion of cilia and it is responsible for rapid and localized NO production (Jackson et al., 2015).

Here we expand our knowledge about the biological function of NO during embryonic epidermis formation in Xenopus laevis. We found out that NO regulates expression of genes which are important for epidermis development. Moreover, we discovered that the production of NO is cell specific and its inhibition leads to defects in MCCs, ionocytes and SSCs development. Furthermore, we showed correlation between NO signalling and Notch pathway. In summary, we propose a novel function of NO during development, regulation of epidermal formation, which is probably necessary for embryonic epidermis development and can be crucial for human mucus and mucociliary epithelium function.

RESULTS

Identification of epidermal cell types producing NO

NO production and nNOS expression were previously identified in the most outer layer of the developing epidermis in the sagittal sections at stage 20 (Jacox et al., 2014). Our goal here is to show detailed analysis of NO and nNOS at different embryonic stages. We performed NO staining at stages 10, 26, 36 and 40 and in situ hybridization using probes against eNOS and nNOS at stages 26 and 30 (Figs 1 and 2). Production of NO is apparent in early developmental stages (stage 10) in Xenopus embryos (Fig. 1A-C). NO staining revealed the ‘salt and pepper’ pattern at stage 26, characteristic for specific epidermal cell-type activity (Fig. 1D). We combined NO staining with immunohistochemistry using antibody markers for MCCs (α-tubulin) and SSCs (serotonin) at stages 26 (Fig. 1E-G), 36 (Fig. 1H-I) and 40 (Fig. 1K-P) to identify the cell type producing NO. Our results showed that the NO is produced in ionocytes throughout the studied early embryonic development (Fig. 1G,J,M,P) and in MCCs at stages 26 and 36 (Fig. 1G,J). NO was not detected in MCCs at stage 40 (Fig. 1M,P). Production of NO was not detected in either SSCs nor goblet cells (Fig. 1P).

Interestingly, expression of eNOS (Fig. 2A,B) and nNOS (Fig. 2C, D) correlates with NO production. Both are expressed in the same ‘salt and pepper’ pattern at stages 26 and 30 (Fig. 2).

NO regulates expression of genes, which are necessary for epidermis development and function

It is known that NO regulates gene expression (Bogdan, 2001; Pfeilschifter et al., 2001). We performed transcriptome comparison between embryos with inhibited NO production and sibling controls. RNA-Seq data contained expression of 28,399 genes at stage 26, 1702 of them showed dependence NO (a statistically significant adjusted P value lower than 0.1 and significant difference higher than 2+), 728 were upregulated, and 974 were downregulated (Fig. S1). Table 1 summarises the top genes which are regulated by NO. Interestingly, lots of downregulated genes were related to epidermis development and function (especially collagens and keratins). We selected 37 candidate genes from transcriptome analysis for validation and performed RT-qPCR (Fig. S1C). Comparison between the results from RNA-Seq and RT-qPCR showed similarity (R²=0.6316).

To test downregulation of collagens at protein levels we performed Masson’s trichrome staining using control and NO-inhibited embryos at stage 26. Our results demonstrate that inhibition of NO lead to decrease in the amount of collagens protein more than 30% (Fig. S2).

Inhibition of NO causes decrease in number of specific epidermal cell types

Production of NO in the epidermis led us to hypothesize that NO is required for epidermis development and cell specific formation. We performed loss-of-function experiments using chemical inhibitors and morpholinos (nNOS MO and eNOS MO). We continued with immunofluorescence staining at stages 30 and 36 using antibody markers for MCCs (α-tubulin) and SSCs (serotonin) (Fig. 3A-H) and in situ hybridization at stage 26 using probes against foxi1e (marker for ionocytes), foxi1a (marker for SSCs), α-tubulin (marker for MCCs) (Fig. 3I-T) and otogelin (marker for goblet cells) at stage 26 (Fig. S3). We compared the numbers of individual epidermal cell types between treated and control embryos. We observed a significant reduction in the number of MCCs, SSCs and ionocytes in embryos with inhibited NO production (Fig. 3D,H,P,T). Immunofluorescence staining showed a decrease in SSCs by 97% (nNOS MO) (Fig. 3F,H) and by 55% (eNOS MO) (Fig. 3G,H) at stage 36. MCCs decreased by 66% (nNOS MO) (Fig. 3B,D) and by 31% (eNOS MO) (Fig. 3C,D) at stage 30 (counted every cell with at least one cilia). Further NO inhibition lead to dramatic decrease of number of cilia and their length (Fig. S4) and phenotypic changes at the level of basal bodies (accumulation of gamma tubulin in the central region of MCCs in embryos with inhibited NO production compared with ctrl MO embryos) (Fig. S5). At least 50% of treated embryos showed this phenotype. Taken together, immunofluorescence staining indicates abnormal ciliogenesis in embryos with inhibited NO production. In situ hybridization for MCCs marker showed decrease by 50% (nNOS MO) (Fig. 3J,L) and by 36% (eNOS MO) at stage 26. SSCs precursors decreased by 85% (nNOS MO) (Fig. 3N,P) and by 77% (eNOS MO) (Fig. 3O,P) at stage 26. Furthermore, we also observed a decrease of ionocytes by 37% (nNOS MO) (Fig. 3R,T) and by 51% (eNOS MO) (Fig. 3S,T) at stage 26. These results indicates that NO is required for the formation of MCCs, SSCs and ionocytes.

Changes in epidermis development were supported also by gene expression analysis using samples of embryonic epidermis at stage 26.
Decrease of other epidermal markers for MCCs (*tuba1a, foxj1*), SSCs (*foxa1a*) and ionocytes (*foxi1e, ATP6V1A*) was found in embryos with inhibited NO and, in contrast, marker of goblet cells (*itln2*) showed increase (Fig. S6).

The NO donor SNAP (S-Nitroso-N-Acetyl-D,L-Penicillamine) caused opposite effects to the loss-of-function experiments – we observed significant increase by 132% in the number of MCCs at stage 30 (Fig. 4B-D). Results from both experiments indicate the necessity of NO for the development of specific epidermal cell types.

**NO acts during epidermis formation through classical sGC-cGMP-PRKG pathway**

One of the most studied mechanisms of NO activity is its activation of sGC followed by the production of cGMP and the subsequent activation of PRKG (Arnold et al., 1977; Francis et al., 2010) (Fig. 4A). We used loss of function of Prkg1 (by prkg1 MO at stage 1, Fig. 4E-G) and specific chemical inhibitor against sGC (ODQ at stage 10, Fig. 4H-J) to determine whether NO regulates epidermis development through the sGC-cGMP-PRKG pathway. Our results showed a significant decrease in the number of MCCs (46% decrease) in the embryos with inhibited prkg1 (Fig. 4F,G) and 87% decrease of MCCs using chemical inhibitor (Fig. 4I,J). This supports mechanism of NO activity through classical sGC-cGMP-PRKG pathway during epidermis development.

**NO is required during early embryonic stages for epidermis development**

Epidermal precursor cells are formed during gastrulation. We tested temporal requirement of NO using the specific chemical inhibitor of NOS called TRIM. We removed manually vitelline membrane and...
treated the embryos with TRIM from stages 10 (early gastrulation), 15 (early neurulation) and 18 (neurulation) until stage 26. Treated embryos were then collected and immunofluorescence staining using antibody markers for MCCs (α-tubulin) was performed (Fig. 5). We found almost complete loss of MCCs in embryos with inhibited NO production by TRIM from stage 10 (95% decrease) (Fig. 5A,A′,E,I). Embryos treated at stage 15 showed 40% decrease in MCCs (Fig. 5B,B′,F,I) and no difference was observed in embryos treated after stage 18 (Fig. 5C,C′,D,D′,G,H,I).

Our data indicates that NO is important for MCCs formation during the early stages of ciliogenesis and its importance disappears after neurulation.

**Notch pathway is connected with NO signalling**

Notch pathway is essential for formation of precursors of the individual epidermal cell types. Exogenous activation of Notch pathway causes reduction of MCCs, SSCs and ionocytes. Whereas Notch pathway inhibition enhances ciliogenesis (shown in *Xenopus* epidermis and human airway epithelium) leading to the multiplication of MCCs precursors and ionocytes (Deblandre et al., 1999; Stubbs et al., 2006; Hayes et al., 2007; Tsao et al., 2009; Marcet et al., 2011; Quigley et al., 2011). Our previous results showing reduction of specific epidermal cell types by NO inhibition indicates possible connection of NO production with Notch signalling. We performed synergy experiments using inhibition of Notch pathway (by *notch1* MO) and inhibition of NO production. Our hypothesis was that Notch inhibition leading to increase of MCCs may reverse the negative effect caused by NO inhibition. We combined *nNOS* MO with *notch1* MO (1:1) and compared it with *nNOS* inhibited embryos and control MO. We used immunohistochemistry staining with antibody markers 5HT/Serotonin for SSCs at stage 30 (Fig. 6A-C) and *in situ* hybridization using probe against *foxi1e* (marker for ionocytes) at stage 40 (Fig. 6D-F). Our results showed significant restoration of the quantity of ionocytes (11% decrease) and SCCs (29% decrease) compared to controls (Fig. 6A,D,G,H). In the same experiment we measured 40% reduction of ionocytes (Fig. 6E,H) and 88% reduction in SCCs (Fig. 6B,G) in *nNOS* loss-of-function embryos. MCCs staining has not shown reproducible significant reversal effect. We conclude from these results that there is connection between Notch pathway and NO production, but more experiments would be needed to reveal the mechanism.

**DISCUSSION**

The NO molecule is a potent biological factor regulating various processes from blood pressure control, inflammation and neurotransmission to gene expression stimulation. NO is usually highly produced in blood vessels and wounded tissues and recently it has been detected in developing epidermis of *Xenopus* (Brunelli et al., 2005; Alpert et al., 2007; Wildling and Kerschbaum, 2007; Jacox et al., 2014) and human airways epithelia (Jiao et al., 2010; Jackson et al., 2015). Surprisingly NO is detected at developmental stages when inflammatory and blood systems are missing. Epidermal production of NO therefore indicates a novel role in early development of this protective layer of embryonic body.

Here, we performed extensive characterization of epidermal NO production and its role during development. We found that NO is produced in ionocytes throughout the early development and in multi-ciliated cells (MCCs) during ciliogenesis. Interestingly, NO was not detected in small secretory cells (SCCs). Our data correlate
nicely with NO production in human airways epithelia, where NO is strongly produced in MCCs (Jackson et al., 2015). Potential importance of NO production in the developing epidermis is further supported by expression of both constitutively active enzymes producing NO [endothelial (eNOS)- and neuronal (nNOS)-NO synthases]. Both enzymes are expressed at the mRNA and protein levels during epidermis formation and create the typical ‘salt and pepper’ pattern similar to the presence of MCC, ionocyte and SSC cells in the developing epidermis. Immunohistochemistry using antibody against nNOS showed the same pattern (data not shown). The third NOS called inducible (iNOS) is expressed weakly during early embryogenesis and we were not able to detect its expression in developing epidermis (data not shown). Expression of iNOS is usually activated following injury as inflammatory response and leads to production of high and toxic levels of NO (Xu et al., 2001). We hypothesize that the physiological level of NO production is usually activated following injury as inflammatory response and leads to production of high and toxic levels of NO (Xu et al., 2001). We hypothesize that the physiological level of NO production is needed for development of epidermis and its function, and that eNOS and nNOS are required for keeping correct NO levels in cells.

Production of NO in the early ionocytes and MCCs indicates the potential role during epidermis development. We performed loss-of-function experiments using gene-specific knock downs of nNOS and eNOS. In both cases, this leads to chronic reduction of NO production and strong phenotypic changes in epidermis. Reduction of NO production had a severe effect on development of MCCs, SSCs, and ionocytes. The main difference was significant reduction of the numbers of these specialized cells. Connection between development of MCCs, SSCs, and ionocytes was shown recently (Dubaissi and Papalopulu, 2011; Dubaissi et al., 2014) and our results support the theory of influence of SSCs and ionocytes on MCCs development. Reduction of cell numbers was higher for loss-of-function compared to eNOS and nNOS and, with lower efficiency, also eNOS (Handy et al., 1996). We found that NO production is required at the very early stages of epidermis specification following gastrulation. Treatment of embryos at the gastrula stage with NOS inhibitor resulted in almost total absence of MCCs development. Inhibition of NO production during later developmental stages showed lower or no effect on MCCs and ionocytes development.

A probable mechanism of NO activity during epidermis development is regulation of gene expression, which is the usual outcome of the physiological production of NO (Pilz et al., 1995; Tapia-Limonchi et al., 2016). We performed transcriptome analysis in embryos with chronic reduction of NO production (Fig. S1). Although we compared transcriptome changes of the whole embryos, a lot of genes with highest altered expression were related to epidermis development and function. We found a reduction of the mRNA levels for members of laminin, keratin, collagen or matrix metalloprotease families, which are considered to be essential for epidermis function. In addition to changes in gene expression, we showed strong reduction in collagen layer in developing epidermis after NO inhibition, which has potential impact on skin function as a barrier (Fig. S2).

Early production of NO in developing epidermis indicates a potential connection with a Notch signalling cascade, which is well known to have a key role for epidermis development. Our preliminary results showed correlation of NO production with Notch signalling, but it is not yet possible to deduce the mechanism of their activities and more thorough studies are required.

The present study highlights the importance of NO during embryonic epidermis formation and its potential for the study of NO production and function in human lung epithelium and related defects.

### Table 1. Gene expression changes in embryos with inhibited NO production relative to control (100%)

<table>
<thead>
<tr>
<th>Genes</th>
<th>RNA-seq results of a subset of genes</th>
<th>RT-qPCR</th>
</tr>
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<tbody>
<tr>
<td>Collagens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>col2a1.S</td>
<td>71% Keratins</td>
<td>col2a1.L</td>
</tr>
<tr>
<td>col3a1.L</td>
<td>21% krt5.6.L</td>
<td>fos1.L</td>
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<td>col3a1.S</td>
<td>33% krt5.7.L</td>
<td>jun.L</td>
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<td>col4a1.S</td>
<td>47% krt12.L</td>
<td>jun.S</td>
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<td>78% krt12.S</td>
<td>junb.1</td>
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<tr>
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<td>46% krt15-like.S</td>
<td>junb.L</td>
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<td>kerat6</td>
</tr>
<tr>
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<td>51% krt19.L</td>
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<td>37% Biglycan</td>
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MATERIAL AND METHODS

Embryos manipulation

Xenopus laevis eggs were fertilized in vitro and cultured in 0.1× Modified Barth’s Saline (MBS: 8.8 mM NaCl, 0.1 mM KCl, 0.5 mM HEPES pH 7.8, 0.07 mM CaCl₂, 0.1 mM MgSO₄, 0.25 mM NaHCO₃)+gentamicin (20 µg/ml) until stage 26-40. Embryos were staged according to the designations established by Nieuwkoop and Faber (1994). Experiments complied with all relevant institutional and national animal welfare laws, guidelines and policies, as should the care and use of experimental animals.

Quantitative real-time PCR

Total RNA from X. laevis embryos was extracted using TRI Reagent® (Sigma-Aldrich, T9424) following their standard protocol. cDNA was then synthesized from 100 ng of total RNA by Superscript III reverse transcriptase (Invitrogen™, 18080093) with thermocycling condition: 75°C for 5 min, 25°C for 20 s, 4°C for 2 min, 25°C for 5 min, 50°C for 60 min, 55°C for 15 min, 75°C for 15 min. qPCR analysis was performed on the C1000™ Thermal Cycler CFX384™ Real-time system (Bio-Rad) with iQ™ SYBR Green Supermix (Bio-Rad, 1708880) using thermocycling condition: initial denaturation at 95°C for 3 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 20 s and elongation at 72°C for 20 s followed by melting curve analysis. Biological triplicate reactions were performed for each condition including negative controls and gDNA contamination controls. Primer sequences are available on request.

Morpholino oligonucleotides

All morpholinos (MO) were purchased from Gene Tools, LLC (Philomath, OR, USA). Standard control MO designed by Gene Tools was used in control injections. Sequence of the nNOS spliced morpholino was obtained from Jacox et al. (2014) (5′-TGGCTAAAAGAACACAGGACATCAA-3′), while the sequence of notch1 MO was obtained from www.xenbase.org (5′-GCACAGCCAGCCCTATCCGATCCAT-3′). Morpholinos for eNOS and...
prkg1 (for α and β variant) were designed and tested before the experiments and the most efficient sequences were found to be 5′-TTCAGCTCAT-GTACCCTACCTGCC-3′ (prkg1 MO) and 5′-AAAAGCCAAGCAGTAC-TCACCCTGTTT-3′ (eNOS MO), respectively. Morpholinos were injected at the one-cell stage unless otherwise stated. MOs were injected at TCACCGTTT-3′ and the most efficient sequences were found to be 5′-AAAAGCCAAGCAGTAC-TCACCCTGTTT-3′ (eNOS MO) and prkg1-AAAAGCCAAGCAGTAC-TCACCCTGTTT-3′ (prkg1 MO) and 5′-AAAAGCCAAGCAGTAC-TCACCCTGTTT-3′ (prkg1 MO) in parallel appropriate amount of control MO.

RNA-Seq

Embryos were injected at the one-cell stage using 17 ng of eNOS MO 17 ng, eNOS MO 34 ng, notch1 MO 17 ng, prkg1 MO 51 ng and in parallel appropriate amount of control MO.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed according to (Sive et al., 2000). Clones of foxa1 (MXL1736-202771841), foxi1e (MXL1736-202771935) and a-tubulin (MXL1736-202771973) were purchased from Dharmaco (Lafayette, Colorado, USA). Full-length coding sequences for eNOS and nNOS were cloned into pBlueScript KS+ vector using primers eNOS (forward ATGGAAGAATATGAGTTCAGC; reverse TTATGAGCAGAAAACCTCATCTGA). The constructs were linearized and transcribed with T7 RNA polymerase (Agilent, 600123-51) for 30 min at room temperature.

Nitric oxide staining

Embryos were incubated in NO-indicator 4-Amino-5-Methylamino-2′,7′-Difluorofluorescein Diacetate (1:200, Life Technologies) for 30 min at room temperature.
(or overnight at 4°C), counterstained using DAPI (1:1000, Sigma-Aldrich 32670-MG-F) and primary antibodies against α-tubulin (1:1000, Sigma-Aldrich T9026) or Serotonin (5HT) (1:500, Merck Millipore AB938). Samples were imaged using Leica TCS SPE confocal microscope. Images were analyzed using LAS AF Lite (version 2.6.0), ImageJ (NIH) and Photoshop (Adobe).

**Collagens analysis**
Paraffin-embedded histological sections were stained using Masson’s trichrome staining kit (Sigma-Aldrich, HT15) for the direct visualization of collagens according to manufacturer’s instructions.

**Immunofluorescence staining**
Immunohistochemistry was performed according to Sive et al. (2000) using primary antibodies against monoclonal α-tubulin (1:1000, Sigma-Aldrich T9026), monoclonal gamma-tubulin (1:1000, Sigma-Aldrich T6557), polyclonal Serotonin (5HT) (1:500, Merck Millipore AB938), Alexa Fluor 488 phalloidin (1:1000, Life Technologies A12379), Alexa 647 goat anti-rabbit (1:250, Life technologies A21244) and Alexa 647 goat anti-mouse (1:500, Life Technologies A21235) were used as secondary antibodies. Samples were imaged using Leica TCS SPE confocal microscope. Images were analyzed using LAS AF Lite, ImageJ (NIH) and Photoshop (Adobe).

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**Fig. 5. Determination of developmental stages for necessity of NO.** The vitelline membrane was removed from embryos at stage 8 to 9. Treatment by TRIM were performed at stage 10 (A,A′,E), 15 (B,B′,F) and 18 (C,C′,G) and all embryos, treated and control (D,D′,H), were collected at stage 26. Embryos were stained with an antibody against α-tubulin (MCCs) and phalloidin (membranes) and imaged (apical surface of the embryonic epidermis) by confocal microscopy (magnification 40×, scale bar: 20 µm). Treatment with TRIM reduced number of MCCs in embryos treated at stage 10 and 15 (A-B). (I) Quantification of incidence of MCCs error bars indicate +s.d., *P<0.03 (ctrl×st. 10, ctrl×st. 15), no statistically significant difference between ctrl embryos and embryos treated by TRIM at stage 18 (C-D). At least five embryos were used for each condition (one-way ANOVA, Tukey’s multiple comparisons test).
NO donor and inhibitor experiments

NO donor, S-Nitroso-N-acetyl-DL-penicillamine (SNAP, Sigma-Aldrich N3398) was diluted to 100 mM in DMSO. 1 nl of SNAP was injected into oocytes. Embryos were collected at tailbud (stage 30) for immunohistochemistry.

NOS inhibitor 1-(2-Trifluoromethylphenyl) imidazole (TRIM, Sigma-Aldrich T7313) was diluted to 1 M concentration in DMSO and embryos at stage 10, 15, 18 and 20 with manually removed vitelline membrane were incubated in solution of 2 µl of ODQ per 1 ml of 0.1× MBS till stage 26. Embryos were then collected and fixed for immunohistochemistry.

The inhibitor of soluble guanylate cyclase, 1H-[1,2,4]Oxadiazolo[4,3-a] quinoxalin-1-one (ODQ, Sigma-Aldrich O3636) was diluted to 100 mM in DMSO and embryos at stage 10 with manually removed vitelline membrane were incubated in solution of 2 µl of ODQ per 1 ml of 0.1× MBS till stage 26. Embryos were then collected and fixed for immunohistochemistry.

Collection of embryonic epidermis

Embryos at stage 26 were decapitated and incubated in papain solution (Merck millipore, L2223) for 15 min. Epidermis was manually collected into TRI Reagent for RNA extraction.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.T.; Software: P.A.; Formal analysis: S.T.; Investigation: P.A.; Writing - original draft: S.T., R.S.; Writing - review & editing: S.T., R.S.; Visualization: S.T.; Supervision: R.S.

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