Irx1 and Irx2 Are Coordinately Expressed and Regulated by Retinoic Acid, TGFβ and FGF Signaling during Chick Hindlimb Development

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Abstract

The Iroquois homeobox (Ir) genes play a crucial role in the regionalization and patterning of tissues and organs during metazoan development. The Irx1 and Irx2 gene expression pattern during hindlimb development has been investigated in different species, but its regulation during hindlimb morphogenesis has not been explored yet. The aim of this study was to evaluate the gene expression pattern of Irx1 and Irx2 as well as their regulation by important regulators of hindlimb development such as retinoic acid (RA), transforming growth factor β (TGFβ) and fibroblast growth factor (FGF) signaling during chick hindlimb development. Irx1 and Irx2 were coordinately expressed in the interdigital tissue, digital primordia, joints and in the boundary between cartilage and non-cartilage tissue. Down-regulation of Irx1 and Irx2 expression at the interdigital tissue coincided with the onset of cell death. RA was found to down-regulate their expression by a bone morphogenetic protein-independent mechanism before any evidence of cell death. Furthermore, TGFβ protein regulated Irx1 and Irx2 in a stage-dependent manner at the interdigital tissue, it inhibited their expression when it was administered to the interdigital tissue at developing stages before their normal down-regulation. TGFβ administered to the interdigital tissue at developing stages after normal down-regulation of Irx1 and Irx2 evidenced that expression of these genes marked the boundary between cartilage tissue and non-cartilage tissue. It was also found that at early stages of hindlimb development FGF signaling inhibited the expression of Irx2. In conclusion, the present study demonstrates that Irx1 and Irx2 are coordinately expressed and regulated during chick embryo hindlimb development as occurs in other species of vertebrates supporting the notion that the genomic architecture of Irx clusters is conserved in vertebrates.

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Introduction

In vertebrate limb development, the outgrowth and patterning of the proximo-distal axis is controlled by the apical ectodermal ridge (AER), a thickened epithelial rimming the distal tip of the growing limb, which secretes FGF to mesenchymal cells located beneath the AER, giving rise to the undifferentiated region of the limb [1,2]. During the hand plate development, the TGFβ/Activin signaling pathway recruits undifferentiated mesenchymal cells to the cartilage lineage, prefiguring digital rays [3]. TGFβ/Activin signaling induces the formation of an ectopic digit by promoting activation of a chondrogenic molecular cascade in which Sox9 expression is induced [4,5,6,7]. In contrast, if undifferentiated mesenchymal cells are not recruited to form digital rays, they develop into interdigital tissue. In species with free digits, the loss of FGF signaling from AER promotes interdigital cell death [8,9]. The inhibition of Fgf8 expression in the AER is under the control of BMP signaling leading to apoptosis [9]. In addition, RA a pro-apoptotic factor, antagonizes FGF8 function inhibiting Fgf receptor-1 gene expression [8].

The Iroquois genes (Ir) encode homeoproteins that belong to the TALE superclass of homeobox transcription factors with an atypical homeodomain and a small conserved C-terminal region in the protein: the Iroquois box [10]. In vertebrates, their genomic organization is in two cognate clusters of three genes each, cluster A includes Irx1, Irx2, and Irx4 and cluster B, Irx3, Irx5, and Irx6 [10]. The gene expression pattern of Irx genes illustrates that members of a same cluster are coordinately expressed during embryonic development [11,12]. They have been involved in the regionalization of territories and patterning; for instance, at early stages of chick embryos, Irx2 expression prefigures a subdivision of the neural plate [13]. In Xenopus embryos, Irx1 is essential to specify the neural territory and dorsal mesoderm by repressing expression of BMP4 [14,15].

In the hand plate development of human, mouse and chick embryos, Irx1 and Irx2 are expressed during digital formation [16]. In mouse limb development, Irx1 expression takes place in the
posterior digital condensation, delimited distally by the undifferentiated zone. Later, this expression expands to anterior digits, and finally it is visible in presumptive joint sites [17]. In chick limb embryo, Irx1 expression is observed only in the digital area [16]. Meanwhile, expression of Irx3, Irx5 and Irx6 occurs in the interdigital tissue of mouse; similarly, expression of Irx6 in chick embryos is clearly observed in the interdigital tissue [16,19]. The mouse mutant Fused toes (Ft) is caused by an autosomal dominant mutation resulting from the deletion of six genes, which include cluster B of the Irx genes [19]. The heterozygous Ft/Ft limb phenotype is characterized by numerous and small condensations at metacarpal level, and syndactyly of fore- and hindlimbs, making the Ft/Ft mouse a useful model to study the role of Irx genes in limb development. In addition, the cell death pattern is altered in the anterior region of the presumptive digit area, in which misexpression of Bmp4, Dkk, Msx1 and Msv2 occurs [20]. On the other hand, the Irx2 mouse mutant develops normally, and because Irx1 and Irx2 are coordinately expressed in the developing limb and other members of the cluster B are expressed during digit development, other Irx genes could possibly compensate its function.

In the present study, the main aim was to determine the gene regulation of Irx1 and Irx2 during chick hindlimb development. In contrast to a previous study that shows that expression of Irx1 occurs in the digit-forming region and later in the digits, and Irx2 expression occurs throughout the digital plate of chick embryo [16]. The present study shows that Irx1 and Irx2 have comparable gene expression patterns during chick hindlimb development, and that they are coordinately expressed in the interdigital tissue and digital primordia, joints and in the boundary between cartilage and non-cartilage tissue. Down-regulation of Irx1 and Irx2 at the interdigital tissue coincides with the onset of cell death. Furthermore, an interesting finding of this study is that RA inhibited Irx1 and Irx2 expression by a BMP-independent mechanism before any evidence of active caspase 3. In addition, since Irx1 and Irx2 expression was observed at digit primordia, this study made use of the experimental model of ectopic digit induction by TGFβ to determine the role of TGFβ in the control of Irx1 and Irx2 expression during digit formation in the interdigital tissue at stages before and after Irx1 and Irx2 down-regulation in the interdigital tissue. At developing stages before down-regulation of Irx1 and Irx2, TGFβ inhibited the expression of the two genes. In contrast, at stages after down-regulation, the expression of these genes was regulated in a concentration-dependent manner. In addition, at early stages the expression of Irx1 and Irx2 was down-regulated by FGF signaling. Although expression of these genes has been previously reported in the development of the chick hindlimb, the present study demonstrates that Irx1 and Irx2 are coordinately expressed and regulated during chick embryo hindlimb development as occurs in mouse, Xenopus and zebrafish embryos lending support to the notion that the genomic architecture of Irx clusters is conserved in vertebrates [12].

Results

Down-Regulation of Irx1 and Irx2 in Interdigital Tissue Coincides with the Onset of Cell Death

Expression of Irx1 and Irx2 was evaluated during hindlimb development from stages 24–31 HH. In this study both genes were coordinately expressed at stage 24–28 HH first in the posterior region and uninterrupted in both prospective digital and interdigital regions (Fig. 1A–D, 1H–K). Here, down-regulation of Irx1 and Irx2 in the third interdigit began at stage 29 HH coinciding with the onset of cell death (Fig. 1E,L,S). At stage 30–31 HH, down-regulation of both genes persisted in the first and second interdigital tissues and eventually disappeared in all interdigits and the presence of active caspase 3 is evident (Fig. 1F–G, M–N, T–U). In accordance with other reports [16,18], Irx1 and Irx2 were expressed in the prospective and presumptive joint sites and in the boundary between cartilage and non-cartilage tissue.

RA Down-Regulates Irx1 and Irx2 Expression Before the Onset of Cell Death

In order to determine whether down-regulation of Irx genes was associated with the onset of cell death in interdigital tissue, here was evaluated the role of RA and BMP on promotion of cell death and on regulation of Irx genes. RA and BMPs are potent promoters of cell death during interdigital regression [21,22,23]. Beads soaked in the pro-apoptotic factors RA and BMP7 were placed in the third interdigit at stage 27 HH. It was observed that after 8 h, RA-treatment began to inhibit Irx1 and Irx2 expression in 9 out of 12 experimental cases (9/12; Fig. 2A–B). Remarkably, this inhibition occurred before the appearance of the first signs of cell death, which were first observed after 12 h of RA-treatment (3/3; Fig. 2C–D). In contrast, BMP7 or NOGGIN did not regulate Irx1 or Irx2 at 8 h (Fig. 2F–G, 2K–L) neither at longer treatments (data not shown). As control of functionality of the proteins, it was observed that cell death was induced by BMP7 (5/6) or inhibited by NOGGIN (7/9), at 8 and 12 h (Fig. 2H–I and 2M–N). To confirm that BMP signaling was not involved in Irx1 and Irx2 regulation induced by RA, one bead soaked in RA and another in NOGGIN were simultaneously placed in the third interdigit. Results showed that under these conditions NOGGIN at 8 h (Fig. 2O–P; 6/8) or up to 24 h (data not shown) never repressed the inhibitory effect of RA on Irx1 and Irx2 expression, as control of functionality of the protein it was observed that cell death promoted by RA was inhibited by NOGGIN at 8 and 12 h, indicating that protein was functionally active (3/4; Fig. 2Q–R). Control beads never induced cell death (Fig. 2E,J).

The expression of Irx1 and Irx2 was observed in skeletal primordia (Fig.1A–N) and as RA is known to inhibit chondrogenesis and promote cell death [22], the role of RA on regulation of Irx1 and Irx2 at the digital rays was evaluated. Results showed that Irx1 and Irx2 expression began to be inhibited from 4 h post-treatment (4/4) before cell death induction (4/4) that was evident from 12 h post-treatment, correlating with Sox9 down-regulation (3/4; Fig. 3).

TGFβ Regulates Irx1 and Irx2 Expression during Chondrogenesis

On the basis that RA and TGFβ have antagonistic functions in the control of chondrogenesis and cell death, and that in the present study the concomitant down-regulation of Irx1, Irx2 and Sox9 expression occurred after RA treatment, the role of TGFβ in the regulation of Irx1 and Irx2 was evaluated in the interdigital tissue during ectopic digit formation [5,7,23] by performing two experiments. The first experiment examined if TGFβ regulated Irx1 and Irx2 expression at developmental stages before normal down-regulation of these genes in the interdigital tissue. Thus, beads soaked in TGFβ were placed in the interdigital tissue of hindlimbs at stage 27 HH. Results showed that inhibition of Irx1 and Irx2 expression began at 4 h of TGFβ treatment (10/10; Fig. 4A–B). Similarly, the second experiment was performed in ectopic digit at 29 HH to explore if TGFβ was able to regulate Irx1 and Irx2 expression, which normally disappeared from the interdigital tissue. Results showed that Irx1 expression began after
Figure 1. Gene expression of *Irx1* and *Irx2* is down-regulated in the interdigital tissue and coincides with the onset of cell death.

Whole mount *in situ* hybridization of *Irx1* (A–G) and *Irx2* (H–N) at stages 24 HH (A, H), 26 HH (B, I), 27 HH (C, J), 28 HH (D, K), 29 HH (E, L), 30 HH (F, M) and 31 HH (G, N), respectively. Note that *Irx1* and *Irx2* are coordinately expressed in the interdigital tissue, digital primordia, joints and in the boundary between cartilage and non-cartilage tissue. *Sox9* expression at stages 24 HH (O), 26 HH (P) and 27 HH (Q) is presented to compare cartilage differentiation with *Irx* expression at developing stages 24 HH, 26 HH and 27 HH. Active Caspase 3 immunolocalization (green) in the third interdigit region of hindlimbs at 28 HH (R), 29 HH (S), 30 HH (T) and 31 HH (U) is presented to compare *Irx* interdigital expression with the cell death pattern. (R–U) Slices were cut at 50 μm. The squares indicated in D, K, E, L, F, M, G, N are to compare with panels in which the activity of caspase 3 was analyzed in R–U. Natural red color of Cy3 used to detect active caspase 3 was changed to green color to obtain a better visualization of the images.

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Figure 2. RA inhibits *Irx1* and *Irx2* expression before the onset of cell death by a BMP-independent mechanism. *Irx1* and *Irx2* expression at stage 27 HH in chick hindlimbs, after RA-treatment (A, B), BMP7-treatment (F, G), NOGGIN-treatment (K, L), and RA/NOGGIN-double treatment (O, P) for 8 h in the interdigital area. Note that *Irx1* and *Irx2* expression is inhibited by RA but not by BMP7. Active Caspase 3 (green) was evaluated in the third interdigit region of hindlimbs after RA treatment at 8 h (C) and 12 h (D) and BMP7 at 8 h (H) and 12 h (I). Note that the first signs of cell death induced by RA were observed at 12 h (C, D); however, BMP7 was able to induce the first signs of cell death at 8 h and it was most abundant at 12 h (H, I). NOGGIN-treatment (M, N), and RA/NOGGIN-double treatment (Q, R) inhibited cell death induced by RA and BMP7. Control beads without RA or BMP7 never induced cell death (E, J). Experimental samples in A, B, F, G, K, L, O, P are presented on the left; controls on the right. Black arrows indicate the area of *Irx1* and *Irx2* inhibition. Autofluorescence in green was observed in ionic-exchange beads used for RA-treatment (black asterisks). The natural red color of Cy3 used to detect active caspase 3 was changed to green for better visualization of the images. (White asterisks indicate beads soaked in BMP7 or NOGGIN).

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in 50 ng/μl TGFβ were placed at the tip of digits at stage 27 HH. We preferred to use this concentration because it had similar effects that 75 ng/μl TGFβ. Under these conditions, the expression of Sox9 in the phalanx was enlarged showing a rounded appearance (3/4; Fig. 6A). Irx1 and Irx2 expression seemed inhibited below the bead from 0 h, while above it the expression was noted as a transversal line in the phalanx (Fig. 6A-C). After 24 h of TGFβ-treatment, Irx1 and Irx2 expression appeared around the enlarged and rounded cartilage (6/9) that is Sox9 positive, resembling a perichondrium (Fig. 6D–E).

Irx1 and Irx2 Expression is Regulated by FGF Signaling

An observation obtained from the Irx1 and Irx2 gene expression pattern is that they were not expressed in the undifferentiated zone beneath AER. It is known that this zone is dependent on FGF signaling. Because in this study Irx1 and Irx2 are coordinately expressed and regulated by RA and TGFβ, we solely evaluated the expression of Irx2 in the undifferentiated zone beneath AER region to determine whether non-expression of Irx2 is a consequence of inhibition by FGF signaling. Beads soaked in the FGR-selective inhibitor SU5402 were placed in the undifferentiated zone beneath AER of hindlimbs at stage 24 HH. This treatment was not able to induce Irx2 expression at 2, 4 h, or later, instead it resulted in activation of cell death that was evident at low level from 2 h but it extended from 4 h (Fig 7A–H). Because this analysis did not allow to determine whether the inhibition of FGF signaling regulate Irx2 expression, we determined whether beads soaked soaked in FGF8 or FGF10, placed in posterior-distal region of hindlimbs at stage 24 HH, have effects on Irx2 expression. Results showed that its expression began to be inhibited by FGF8 (3/3) and FGF10 (4/4) from 4 h and 8 h, respectively (Fig. 7I–J).

Discussion

Findings reported by McDonald et al [16] show the expression of Irx1 present in the digit-forming region and later in the digits, while Irx2 is highly expressed throughout the digital plate of the chick embryo. In contrast with that study, the present study showed that Irx1 and Irx2 were coordinately expressed during hindlimb development, as shown in other animal models [12,18]. The two genes were expressed as a continuous label comprising well-defined areas of mesodermal tissue of both prospective digital and interdigital areas, and later in digital and interdigital regions. In fact, the Irx2 probe used in the present study was also used in the mentioned report [16]. Probably, the high concentrations of protease K and the elimination of the Iro and homeo box from both probes allowed us to obtain a specific expression of both genes in hindlimb development.

Here was observed that down-regulation of Irx1 and Irx2 expression in interdigital tissue coincided with the commencement of cell death. However, an interesting finding is that in the interdigital tissue, RA inhibited Irx1 and Irx2 expression before the first signs of active caspase 3 were observed. The role of RA in programmed cell death control during hindlimb development is well known, as well as its effects mediated by BMP signaling [8,22,24,25]. In the present study, RA inhibited Irx1 and Irx2 expression by a BMP-independent mechanism. On this basis, the present study suggests that Irx1 and Irx2 expression may play a protective role against cell death (Fig. 8). It follows that down-regulation of Irx genes in the interdigital tissue by RA may be a pre-requisite to promote the molecular cascade that ends in cell death, since Irx genes may repress the expression of genes involved in the cell death process, as has been suggested for the Msx gene in the boundary between body wall and wing in Drosophila, and for...
Figure 4. TGFβ signaling regulates Irx1 and Irx2 in a stage-dependent manner in the interdigital tissue. Irx1 and Irx2 expression after 4 h of TGFβ treatment at stage 27 HH (A, B). Regulation of Irx1 at 21 h (C), Irx2 at 19 h (D), Sox9 at 30 min (E) and Coll1 at 16 h (F) after TGFβ treatment in the interdigital area at stage 29 HH. An ectopic digit is formed after 4 days of TGFβ treatment (G). Note that TGFβ inhibits Irx1 and Irx2 expression at developing stages at which these genes are still expressed in the interdigital tissue (arrows in A, B). In contrast, TGFβ induces Irx1 and Irx2 expression away and around the bead (arrows in C, D) at developing stages at which Irx1 and Irx2 expression has been down-regulated in the interdigital tissue. doi:10.1371/journal.pone.0058549.g004

Figure 5. Irx1 and Irx2 expression is regulated in a concentration-dependent manner by TGFβ. Irx1 and Irx2 expression at stage 29 HH in chick hindlimbs after 21 hours of TGFβ treatment at different doses 12.5 ng/μl (A, E) 25 ng/μl (B, F) 50 ng/μl (C, G) 75 ng/μl (D, H), respectively in the interdigital area. The black arrows show the distance of Irx1 and Irx2 induction. doi:10.1371/journal.pone.0058549.g005

Bmp4 during Xenopus development [14,15,20,26]. This protector effect of Irx against cell death is probably lost in the limbs of fused-toes mutant mice, which show massive cell death and up-regulation of Bmp4 and Dkk1, as well as down-regulation of Fgf8 and Fgfr2b [20]. Interestingly, Irx3, Irx5 and Irx6 are expressed in the interdigital tissue of the mouse limb as occurs for the Irx1 and Irx2 genes in the chick embryo. So, it is possible that Irx genes may be involved in cell death control. Supporting this hypothesis, in other systems the deletion of Irx genes results in cell death, for instance, Irx1a, Irx4a or Irx1l morphants show extensive cell death in the rostral region of the zebrafish embryo [27], in the CNS [28] and in the developing head and trunk along the neural tube [29], respectively. Moreover, antisense knockdown of pulmonary Irx1, Irx2, Irx3, and Irx5 together increases apoptosis in the mesenchymal compartment of rat lung explants [30].

In this study, the expression of Irx1 and Irx2 was observed in the boundary of skeletal primordia and non-cartilage tissue and in these regions RA also inhibited Irx1 and Irx2 expression concomitantly with down-regulation of Sox9, before induction of cell death. Because it is known that RA is an important inhibitor of chondrogenesis [22], and on the basis that the products of the Irx genes repress Bmp4 or Msx1 expression in other systems, two genes involved in cell death during limb development [14,15,20,26], it may be considered that Irx gene expression during digital formation protects cells committed to the chondrocyte lineage against cell death-promoting signals, delimiting the boundaries of skeletal elements (Fig. 8). It is important to mention that the temporal gene expression of Irx genes (around 21 h) and Sox9 [at 30 min] during ectopic digit formation induced by TGFβ, and the concomitant down-regulation of Irx1, Irx2 and Sox9 expression, which occurred after the first 4 h of RA treatment, suggest that Irx genes are not needed for chondrogenesis.

On the other hand, depending on the developmental stage, TGFβ was also able to regulate Irx1 and Irx2 expression in the interdigital tissue. At developing stages before down-regulation of Irx1 and Irx2, TGFβ produced a halo of inhibition. Whereas, at developing stages after their down-regulation, Irx1 and Irx2 expression appeared to be limited to areas away from the bead and around the expression of Sox9 forming a boundary between non-cartilage tissue and cartilage. Interestingly, it was demonstrated herein that these effects of TGFβ on Irx gene expression are concentration-dependent. Moreover, when TGFβ was placed at the tip of the digits, after the first 8 h of treatment the expression of Irx1 and Irx2 was observed as a transverse line that disappeared after 24 h of treatment. This may reflect that a first wavefront of Irx gene expression inhibition by TGFβ is working, as observed in the interdigital tissue at stages at which Irx genes are present in this tissue. Later, a wavefront of differentiation induced by TGFβ may restrict Irx gene expression to the edges of cartilage. On this basis, it is reasonable to propose that expression of Irx1 and Irx2 during digit development delimits the boundary between cartilage tissue...
and non-cartilage tissue. The establishment of this boundary may reflect the range of diffusion of TGFβ to promote chondrogenesis, which may be counteracted by a chondrogenesis inhibitor, as suggested elsewhere [31,32,33]. Another interpretation is that Irx1 and Irx2 expression at this site could depend on a threshold [34], presumably of TGFβ. These interactions may conceivably allow the formation of the prospective perichondrium.

Another interesting finding was that the Irx1 and Irx2 gene expression patterns were excluded from the undifferentiated zone beneath AER. Furthermore, it was not possible to determine the regulation of Irx2 expression by inhibition of FGF signaling because cell death was induced from 2 h and at later times it was more extended, making difficult to determine the regulation of Irx2 by FGF signaling. However, the treatment with FGF proteins indicated that Irx2 expression began to be inhibited by FGF8 and FGF10 from 4 h and 8 h, respectively. On the other hand, it has previously been demonstrated that during limb development FGF10 induces expression of FGF8, while FGF8 is not able to induce FGF10 [35]. On this basis it is reasonable to speculate that Irx2 gene expression pattern is excluded from the undifferentiated zone beneath AER by action of FGF8 that inhibits expression of Irx2, while inhibition by FGF10 observed in this study may be mediated by FGF8 (Fig. 8).

Finally, this work shows that Irx1 and Irx2 were coordinately expressed and regulated by RA, TGFβ and FGF signaling during chick hindlimb development. Tena et al [12] suggested that coordinate regulation of members of the Iroquois family in mouse, Xenopus and zebrafish is under the control of a genomic architecture. This generates a 3D conformation that allows the promoters of the genes of each cluster are to be located in close proximity [12]. These authors suggest the establishment of regulatory landscapes for certain genes of the cluster in a tissue- and stage-dependent manner [12]. The demonstration in the current study that the coordinated regulation of Irx1 and Irx2 expression by RA, TGFβ and FGF signaling during hindlimb development in chick embryos, supports the notion that the expression patterns are a result of this 3D conformation.
genomic architecture of Irx clusters is conserved in vertebrates [12].

Materials and Methods

Ethics Statement

This research protocol was reviewed and approved by the Institutional Review Board for the Care and Use of Laboratory Animals of Instituto de Investigaciones Biomedicas, UNAM.

Eggs and Embryo Manipulations

Fertilized White Leghorn chicken eggs (ALPES, Puebla, Mexico) were incubated at 38°C and the embryos staged according to Hamburger and Hamilton (HH) [36]. To manipulate embryos, the fertilized eggs were windowed at stage 27–29 HH and the right leg bud was exposed for experimentation. Affigel (Bio-Rad Laboratories, Hercules, CA) and heparin acrylic beads (Sigma-Aldrich) were soaked in 5 mg/ml RA (Sigma-Aldrich) or (Peprotech, Mexico City, Mexico). AGI-X2 ionic exchange beads (Sigma-Aldrich) were soaked in 20 mM SU5402 (Calbiochem, Billerica, MA, USA). Treatments (Sigma-Aldrich) were soaked in 5 mg/ml RA (Sigma-Aldrich) or (Peprotech, Mexico City, Mexico). AGI-X2 ionic exchange beads (Sigma-Aldrich) were soaked in 20 mM SU5402 (Calbiochem, Billerica, MA, USA). Treatments (Sigma-Aldrich) were soaked in 5 mg/ml RA (Sigma-Aldrich) or (Peprotech, Mexico City, Mexico). AGI-X2 ionic exchange beads (Sigma-Aldrich) were soaked in 20 mM SU5402 (Calbiochem, Billerica, MA, USA). 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Author Contributions
Conceived and designed the experiments: MEDH JCM. Performed the experiments: MEDH MB CIGH. Analyzed the data: MEDH MB CIGH.

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