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Published in:
Zygote

DOI:
[10.1017/S096719941800045X](https://doi.org/10.1017/S096719941800045X)

Publication date:
2018

Document Version
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

Citation for published version (APA):

Zhang, X-T., Wang, G., Li, Y., Chuai, M., Lee, K. K. H., & Yang, X. (2018). Role of FGF signalling in neural crest cell migration during early chick embryo development. *Zygote*, 26(6), 457-464.
<https://doi.org/10.1017/S096719941800045X>

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Role of FGF signaling in neural crest cell migration during early chick embryo development.

Xiao-tan Zhang¹, Guang Wang¹, Yan Li¹, Manli Chuai², Kenneth Ka Ho Lee³,
Xuesong Yang^{1*},

¹*Division of Histology & Embryology, Key Laboratory for Regenerative Medicine of the Ministry of Education, Medical College, Jinan University, Guangzhou 510632, China*

²*Division of Cell and Developmental Biology, University of Dundee, Dundee, DD1 5EH, UK*

³*Joint CUHK-UoS Laboratory for Stem Cell and Regenerative Medicine, School of Biomedical Sciences, Chinese University of Hong Kong, Shatin, Hong Kong*

***The corresponding author:** *Department of Histology and Embryology School of Medicine Jinan University No.601 Huangpu Road West Guangzhou 510632, China.*

Fax number: 020-85221343.E-mail: { [HYPERLINK](#)

"mailto:yang_xuesong@126.com" }

Running title: *FGF signaling in chick neural crest cell production*

Financial supports: *NSFC grant (31401230; 81571436); Science and Technology Planning Project of Guangdong Province (2014A020213008) Science and Technology Program of Guangzhou (201510010073); The Fundamental Research Funds for the Central Universities (21615421).*

This is the accepted manuscript version of Zhang, X-T, Wang, G, Li, Y, Chuai, M, Lee, KKH & Yang, X 2018, 'Role of FGF signalling in neural crest cell migration during early chick embryo development' *Zygote* (Cambridge, England), pp. 1-8. <https://doi.org/10.1017/S096719941800045X>

Abstract

FGF signaling acts as one of modulators that control neural crest cell (NCC) migration, but how this is achieved is still unclear. In this study, we investigated the effects of FGF signaling on NCC migration by blocking this process. Constructs that were capable of inducing Sprouty2 (Spry2) or dominant negative FGFR1 (Dn-FGFR1) expressions were transfected into the cells making up the neural tubes. Our results revealed that blocking FGF signaling at stage HH10 (neurulation stage) could enhance NCCs migration at both cranial and trunk levels in the developing embryos. It was established that FGF-mediated NCC migration was not due to alter the expression of N-Cadherin in the neural tube. Instead, we determined that Cyclin D1 was over expressed in the cranial and trunk levels when Sprouty2 was upregulated in the dorsal neural tube. The results imply that the cell cycle was a target of FGF signaling through which it regulates NCC migration at the neurulation stage.

Key words: neural crest cells; FGF signaling; Sprouty2; EMT; cell cycle

Introduction

Neural crest cells (NCCs) are multipotent cells derived from the dorsal side of the neural tube during early embryo development. These NCCs undergo a series of developmental processes that include induction, epithelial-mesenchymal transition (EMT), migration, differentiation, and eventually give rise to cellular components in most vertebrate organ systems { ADDIN EN.CITE <EndNote><Cite><Author>Hall</Author><Year>2008</Year><RecNum>1</RecNum><DisplayText>(Hall, 2008)</DisplayText><record><rec-number>1</rec-number><foreign-keys><key app="EN" db-id="rddpft2d00v959eexd5pt2abd0szxs5xe9aw">1</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Hall, B. K.</author></authors></contributors><auth-address>Department of

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 url>http://www.ncbi.nlm.nih.gov/pubmed/19179766</url></related-urls></urls></rec
 ord></Cite></EndNote>}. The NCCs delaminate from the neuroepithelium and
 migrate to their predetermined locations. This migration process is tightly regulated to
 allow the NCCs to reach their precise target locations - where failure to do so would
 result in birth malformations. This has already been reported for abnormal migration
 of cephalic NCCs in chick embryos { ADDIN EN.CITE
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-type name="Journal Article">17</ref-type><contributors><authors><author>McKinney, M. C.</author><author>McLennan, R.</author><author>Kulesa, P. M.</author></authors></contributors><auth-address>Stowers Institute for Medical Research, 1000 E. 50th St., Kansas City, MO, 64110, USA.Stowers Institute for Medical Research, 1000 E. 50th St., Kansas City, MO, 64110, USA. pmk@stowers.org.Department of Anatomy and Cell Biology, University of Kansas School of Medicine, Kansas City, KS, 64157, USA. pmk@stowers.org.</auth-address><titles><title>Angiopoietin 2 signaling plays a critical role in neural crest cell migration</title><secondary-title>BMC Biol</secondary-title><alt-title>BMC biology</alt-title></titles><pages>111</pages><volume>14</volume><number>1</number><dates><year>2016</year><pub-dates><date>Dec 15</date></pub-dates></dates><isbn>1741-7007 (Electronic)1741-7007 (Linking)</isbn><accession-num>27978830</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/27978830</url></related-urls></urls><custom2>5159958</custom2><electronic-resource-num>10.1186/s12915-016-0323-9</electronic-resource-num></record></Cite></EndNote>}. The timing for cranial NCCs migration is different between chick, mouse and xenopus. In the chick embryo, neural crest migration occurs concomitantly with the fusion of the neural folds. However, in mouse and xenopus embryos, the NCCs migrate when the neural plate is still open { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. The migration of NCCs at the borders of the neural plate/tube relies on inductive signaling molecules from the surrounding neuroepithelium, neural plate and underlying mesoderm { ADDIN EN.CITE <EndNote><Cite><Author>Mayor</Author><Year>1995</Year><RecNum>9</RecNum><DisplayText>(Mayor et al., 1995)</DisplayText><record><rec-number>9</rec-number><foreign-keys><key app="EN" db-id="rddpft2d00v959eexd5pt2abd0szxs5xe9aw">9</key></foreign-keys><ref-type

name="Journal Article">17</ref-type><contributors><authors><author>Mayor, R.</author><author>Morgan, R.</author><author>Sargent, M. G.</author></authors></contributors><auth-address>Laboratory of Developmental Biology, National Institute for Medical Research, Mill Hill, London, UK.</auth-address><titles><title>Induction of the prospective neural crest of *Xenopus*</title><secondary-title>Development</secondary-title><alt-title>Development</alt-title></titles><periodical><full-title>Development</full-title><abbr-1>Development</abbr-1></periodical><alt-periodical><full-title>Development</full-title><abbr-1>Development</abbr-1></alt-periodical><pages>767-77</pages><volume>121</volume><number>3</number><keywords><keyword>Amino Acid Sequence</keyword><keyword>Animals</keyword><keyword>Blastocyst/physiology</keyword><keyword>Carrier Proteins</keyword><keyword>Ectoderm/drug effects</keyword><keyword>Embryonic Induction/*genetics</keyword><keyword>Female</keyword><keyword>Fibroblast Growth Factor 2/pharmacology</keyword><keyword>Gastrula/physiology</keyword><keyword>Gene Expression/drug effects/radiation effects</keyword><keyword>Mesoderm/*physiology</keyword><keyword>Molecular Sequence Data</keyword><keyword>Neural Crest/*physiology</keyword><keyword>Proteins/genetics/pharmacology</keyword><keyword>Ultraviolet Rays</keyword><keyword>Xenopus/*embryology/genetics</keyword><keyword>Zinc Fingers</keyword></keywords><dates><year>1995</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>0950-1991 (Print)0950-1991 (Linking)</isbn><accession-num>7720581</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/7720581</url></related-urls></urls></record></Cite></EndNote>}. The signals involved may include fibroblast growth factors (FGFs), bone morphogenetic protein (BMP), wingless-type (Wnt) and retinoic acid (RA). Normally, a gradient of BMPs is setup at the borders of the neural plate and has

been deemed to be essential for the specification of the developmental fate of the NCCs. The BMPs interact with Wnts, FGFs and retinoic acid to convert the cells at neural plate/tube borders to become neural crest precursor cells { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. These precursors, located at the dorsal-side of the neural tube, then undergo EMT which converts them into mesenchyme-like neural crest cells. This EMT process is modulated by transcription factors, such as members of the Snail, Sox, and Endothelins (Ets) gene families. These transcription factors synergistically regulate cell–cell and cell–matrix adhesions, which facilitates the NCCs to detach from the neuroepithelium ({ ADDIN EN.CITE

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The FGF ligand family is composed of 18 members (FGF1 - 10 and FGF16 - 23), which can be divided into six subfamilies, five paracrine subfamilies, and one endocrine subfamily - according to sequence similarity and phylogeny analysis {

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Andrew.Beenken@med.nyu.edu</auth-address><titles><title>The FGF family:

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 NCC migration is now widely accepted but the difference in NCC migration between
 different animal models should not be ignored { ADDIN EN.CITE
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 om2>3541687</custom2><electronic-resource-num>10.1016/j.ydbio.2012.09.006</e
 lectronic-resource-num></record></Cite></EndNote>}. FGF signaling might exert
 different effects on NCC migration at the cranial and trunk levels due to differences in
 their spatial-temporal bioactivities. For example, FGF signaling is involved in
 regulating frontal bone formation by synergistically working with TGF β and
 dysfunction in this process disrupts cranial NCC osteogenesis, causing abnormal
 craniofacial development { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Li et
 al. have reported that FGF signaling activates NCC proliferation and acquisition of
 osteogenic potential to promote frontal bone development { ADDIN EN.CITE {
 ADDIN EN.CITE.DATA }}. In addition, combined FGF and BMP signalings
 modulate the development of outflow tract valve primordium of heart by directing the
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differentiation of cardiac NCC-derived cushions { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Nevertheless, it is still unclear which role FGFs play during NCC migration in the embryo. In this study, we have employed various approaches to block FGF signaling to elucidate the cellular and molecular mechanism of FGF's action on NCC migration during early chick embryo development.

Materials and Methods

Chick embryos and gene transfection

Fertilized leghorn eggs were acquired from the Avian Farm of South China Agriculture University. They were incubated in a humidified incubator (Yiheng Instruments, Shanghai, China) set at 38°C with 70% humidity. The eggs were incubated until the chick embryos reached the desired developmental stage. For gene transfection, 0.5µl of plasmid DNAs (1.5 mg/ml *GFP*, *Sprouty2-GFP* or *Dn-FGFR1-GFP*) { ADDIN EN.CITE { ADDIN EN.CITE.DATA }} were microinjected into the neural tubes of HH10 (Hamburger and Hamilton stage 10) chick embryos { ADDIN EN.CITE <EndNote><Cite><Author>Hamburger</Author><Year>1951</Year><RecNum>267</RecNum><DisplayText>(Hamburger and Hamilton, 1951)</DisplayText><record><rec-number>267</rec-number><foreign-keys><key app="EN" db-id="rddpft2d00v959eexd5pt2abd0szxs5xe9aw">267</key></foreign-keys><ref-type pe name="Journal Article">17</ref-type><contributors><authors><author>Hamburger, V.</author><author>Hamilton, H. L.</author></authors></contributors><titles><title>A series of normal stages in the development of the chick embryo</title><secondary-title>J Morphol</secondary-title><alt-title>Journal of morphology</alt-title></titles><periodical><full-title>J Morphol</full-title><abbr-1>Journal of morphology</abbr-1></periodical><alt-periodical><full-title>J

Morphol</full-title><abbr-1>Journal of morphology</abbr-1></alt-periodical><pages>49-92</pages><volume>88</volume><number>1</number><keywords><keyword>Animals</keyword><keyword>Chick Embryo</keyword><keyword>*Embryo, Mammalian</keyword><keyword>*Embryo, Nonmammalian</keyword></keywords><dates><year>1951</year><pub-dates><date>Jan</date></pub-dates></dates><isbn>0362-2525 (Print)0022-2887 (Linking)</isbn><accession-num>24539719</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24539719</url></related-urls></urls></record></Cite></EndNote>}. Using a pair of platinum wires fashioned in parallel as electrodes, the plasmids carrying our gene of interest was randomly transfected into the neural tube cells by electroporation. The parameters used for efficient electroporation was as previously described { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. For one-sided gene transfection, the polarity of the electric pulse was kept constant (Supplementary Fig 1). After electroporation, the embryos were further incubated for 8-10 hours. At the experiments, all embryos were photographed and fixed for immunofluorescent staining and in situ hybridization. All experiments were performed in replicates of at least four embryos. All animal experiments were performed according to relevant national and international guidelines and approved by the Medical Research Animal Ethics Committee at Jinan University.

Immunohistological staining

Immunofluorescent staining was performed on whole-mount embryos using HNK1, PAX7, and N-Cadherin antibodies, according to methods previously described { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Briefly, the embryos were fixed in 4% paraformaldehyde at 4°C overnight and then washed with PBS. Unspecific immunoreactions were blocked using 2% Bovine Serum Albumin + 1% Triton-X + 1% Tween 20 in PBS, for 2 hours at room temperature. The embryos were then washed in PBS and incubated with primary monoclonal HNK1 (C0678, Sigma-Aldrich, 1:200, PAX7 (pax7, DSHB, 1:100) and N-Cadherin (6B3, DSHB, [键入文字]

1:100) antibodies, overnight at 4°C on a shaker. After extensive washing, the embryos were incubated with anti-mouse or -rabbit Alexa Fluor 555 antibodies (2µg/ml, Invitrogen) overnight at 4°C. After immunofluorescent staining, all the embryos were counterstained with DAPI (Invitrogen, 1:1000) for 30min at room temperature. All immunofluorescent staining was performed in replicates from at least 4 embryos.

In situ hybridization

Whole-mount in situ hybridization of chick embryos was performed according to methods previously described (Henrique et al., 1995). Digoxigenin-labeled RNA probes were synthesized to detect the presence of Cyclin D1 transcripts in cells (Shoval et al., 2007). The whole-mount stained embryos were photographed and then frozen sections were prepared from them by cutting at thickness of 15µm on a cryostat microtome (Leica CM1900).

Semi-quantitative RT-PCR

Total RNA was isolated from embryonic tissues using a Trizol kit (Invitrogen, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized, to a final volume of 25µl, using SuperScript III First-Strand (Invitrogen, USA). Following reverse transcription, PCR amplification of the cDNA was performed using as follows: N-Cadherin: 5'- CTGGGGACATTGGGGACTTC-3', 5'-ATAGTCTTGCTCACCACCGC-3' { ADDIN EN.CITE { ADDIN EN.CITE.DATA }} . PCR was performed in a Bio-Rad S1000TM Thermal cycler (Bio-Rad, USA). The cDNAs were amplified for 30 cycles. One round of amplification was performed at 98°C for 10 sec, at 60°C for 15 sec and at 72°C for 30 sec (TaKaRa, Japan). The PCR products (20 µl) were resolved on 1% agarose gels (Biowest, Spain) in 1× TAE buffer (0.04 M Trisacetate and 0.001 M EDTA), and GeneGreen Nucleic Acid Dye (TIANGEN, China). The reaction products were visualized using a transilluminator (SYNGENE, UK) and a computer-assisted gel documentation system (SYNGENE). Each of these experiments was replicated three times.

Photography

After immunohistological staining, the whole-mount chick embryos were photographed using a fluorescence stereomicroscope (MVX10; Olympus, Osaka, Japan) and imaging software (Image-Pro Plus 7.0). Sections of the stained embryos were photographed using an epifluorescence microscope (Olympus IX51, Leica DM 4000B) at 200× or 400× magnification using the Olympus software package (Leica CW4000 FISH).

Data analysis

Areas that positively expressed HNK1 and PAX7 were measured and quantified using an Image-Pro Plus 7.0. Data analyses and statistical charts were constructed using a Graphpad Prism 5 software package (Graphpad Software, CA, USA). The results were presented as mean value ($\bar{x} \pm SE$). All data were analyzed using ANOVA, which was employed to establish whether or not there was any difference between control and experimental data. $P < 0.05$ was significantly different.

Results

Inhibition of FGF signaling by Sprouty2 in the developing neural tube increases NCC migration.

We investigated whether FGF signaling was involved in NCC migration during development. This was addressed by transfecting Sprouty2-GFP, which negatively regulate FGF signaling { ADDIN EN.CITE <EndNote><Cite><Author>Aranda</Author><Year>2008</Year><RecNum>271</RecNum><DisplayText>(Aranda et al., 2008)</DisplayText><record><rec-number>271</rec-number><foreign-keys><key app="EN" db-id="rddpft2d00v959eexd5pt2abd0szxs5xe9aw">271</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Aranda, S.</author><author>Alvarez, M.</author><author>Turro,

S. Laguna, A. de la Luna, S. & Dis Program, Barcelona 08003, Spain; Ctr Invest Biomed Red Enfermedades Raras, CIBERER ISCIII, Barcelona, Spain; ICREA, Barcelona, Spain

Sprouty2-mediated inhibition of fibroblast growth factor signaling is modulated by the protein kinase DYRK1A

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}, into one half of the neural tube in HH10 chick embryos. Transverse sections of the cranial and trunk regions of the transfected embryos confirmed that the left side of the neural tubes was transfected and expressed GFP (control plasmid) or Sprouty2-GFP. The right side of the neural tube did not express GFP and served as the un-transfected control (Fig. 1A1 - D1). Immunofluorescent staining was performed on the transfected embryos using HNK1 antibody to reveal the presence of the migrating NCCs. The results showed that inducing Sprouty2-GFP expression in the neural tube caused a significant increase in

HNK1⁺ NCCs, at both the cranial and trunk levels (Fig. 1B2 - 3 and D2 - 3) compared with neural tubes transfected with GFP alone (Fig. 1A2 - 3 and C2 - 3). The increase in NCCs induced by Sprouty2, compared with the control, was observed in 12 out of 14 transfected experimental embryos examined (Control = 0%, N = 0/8; Sprouty2-GFP = 85.71%, N = 12/14) (Fig. 1E). The increased presence of HNK1⁺ NCCs was statistically significant at both cranial and trunk regions (Cranial: 1.65 ± 0.55 , $p=0.007$; Trunk: 1.95 ± 0.76 , $p=0.003$; N=8) as compared with the control (Cranial: 1.05 ± 0.15 ; Trunk: 1.01 ± 0.03 ; N = 8) (Fig. 1F).

To verify the above observations, the transfected embryos were immunofluorescently stained with PAX7 antibody which reveals the presence of pre-migratory NCCs (Supplementary Fig 2). In the control, GFP transfection alone did not affect the symmetrical distribution of PAX7⁺ NCCs at cranial level (Supplementary Fig 2A2 - 3). In contrast, Sprouty2-GFP transfection increased the production of cranial PAX7⁺ NCCs (Supplementary Fig 2B2 - 3) in 72.73% of experimental embryos examined (control = 0%, N = 0/8; Sprouty2-GFP = 72.73%, N = 8/11) (Supplementary Fig 2C). This increase in cranial NCCs induced by Sprouty2 was statistically significant (Cranial: 2.08 ± 0.30 , $p=0.007$; N=5) compared with the control (Cranial: 1.00 ± 0.02 ; N=5) (Supplementary Fig 2D). The results suggest that blocking FGF signaling with Sprouty2-GFP can increase the number of pre-migratory NCCs at both cranial and trunk levels.

Effects of blocking FGFR1 on NCC migration

FGF signaling in the neural tube can be blocked by transfecting a dominant negative FGFR1 (Dn-FGFR1) construct into the neural tubes. We have transfected half-a-side (left-hand-side) of the neural tube with the Dn-FGFR1 construct, which was confirmed by co-transfection with a GFP construct (Fig. 2A1 - D1). We first investigated whether the number of HNK1⁺ NCCs was increased by Dn-FGFR1 blocking FGF signaling. The results revealed that there were significantly more HNK1⁺ NCCs at the Dn-FGFR1-transfected side than the contralateral control side, at both cranial and trunk levels (Fig. 2B2 - 3 and D2 - 3). This increase was not

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observed when the neural tube was transfected with GFP instead of Dn-FGFR1 (Fig. 2A2 – 3 and C2 - 3). The increase in NCCs induced by blocking FGFR1 was observed in 71.43% of embryos examined (control = 0%, N = 0/8; Dn-FGFR1= 71.43%, N = 10/14) (Fig. 2E). The increase of HNK1⁺ NCCs was statistically significant (Cranial: 1.85 ± 0.70 , $p=0.009$; Trunk: 2.05 ± 0.88 , $p=0.005$; N = 5) compared the controls (Cranial: 1.05 ± 0.15 ; Trunk: 1.01 ± 0.03 ; N = 5) (Fig. 2F).

Effects of inhibiting FGF signaling by Sprouty2 on N-cadherin expression in the neural tube

Immunofluorescent staining for N-Cadherin (Fig. 3A2 - D2) was performed on embryos that have been transfected with Sprouty2-GFP on half-a-side of the neural tube (Fig. 3A1- D1). The results showed that inducing Sprouty2 expression in the neural tube did not affect N-Cadherin expression at both cranial and trunk levels (Fig. 3A2 -D2 and A3 - D3). There was no statistical difference in N-Cadherin expression between the control and Sprouty2 groups (Cranial: 1.01 ± 0.11 , $p=0.944$; Trunk: 1.06 ± 0.12 , $p=0.430$; N = 5) (Fig. 3F). The results suggest that suppressing FGF signaling in the neural tube will not alter N-Cadherin expression.

Effects of inhibiting FGF signaling by Sprouty2 on Cyclin D1 expression in the neural tube

We performed whole-mount *in situ* hybridization for Cyclin D1 expression on embryos transfected with GFP or Sprouty2-GFP on half-a-side of the neural tube (Fig. 4A1-D1). We determined that the area of neural tube expressing Cyclin D1 was significantly increased by Sprouty2-GFP (Fig. 4B2 and D2) as compared with the GFP control (Fig. 4A2 and C2). The results imply that inhibiting FGF signaling by Sprouty2 increased cell proliferation which was observed in 73.68% (n=14/19) of experimental embryos examined Fig. 4E and F).

Discussions

Neural crest cell migration is an essential morphogenetic process in the
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developing embryo. This process has been commonly studied using mouse and zebrafish embryos. However, we selected the early chick embryos as our experimental model because these embryos are easy to manipulate and analyze. In addition, development of chicken embryos at early stages has a lot in common with early human embryos. To date the best live imaging of NCC migration has been conducted with chicken embryos. Hence, the early chick embryo is the most ideal model for investigating NNC migration.

The molecular mechanism controlling NCC migration involves a complex network of interacting genes which is still poorly understood. In human, there are 22 members in the FGF family and some of them are involved in regulating NCC migration by FGF signaling. FGF signaling could be blocked using various molecular approaches such as inducing Sprouty2 expression or inhibiting FGFR1 in the developing neural tube. Moreover, HNK1 and PAX7 expressions could be used to follow NCC migration. NCCs start expressing HNK1 as soon as they delaminate and migrate away from the neural tube, while PAX7 is expressed in pre-migratory NNCs. In this study, we demonstrated that they had the same effects on the HNK1⁺ migrating NCCs production and PAX7⁺ pre-migratory NCCs.

In mammals, Sprouty2 regulates the tyrosine kinase/ERK signaling pathways, including EGF and FGFs. Hence, Sprouty2 is involved in many biological processes, such as cell growth, differentiation and migration { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Our results revealed that blocking FGF signaling significantly increased the number of HNK1⁺ NCCs present at both the cranial and trunk levels in the chick embryo. This result was further validated by transfecting the left-side of the neural tube with GFP (serving as the control) while the right-side with Sprouty2-GFP within the same embryo – to prevent inter embryo variations. HNK1 is mainly expressed by migrating NCCs, so we re-assessed the results by examining the pre-migratory NCCs population which express PAX7 { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. We determined that blocking FGF signaling by inducing Sprouty2 expression in the neural tube also significantly increased the number PAX7⁺ pre-migratory NCCs. Using the same strategy, we transfected [键入文字]

dominant negative FGFR1 (Dn-FGFR1) constructs into the developing neural tube to block FGF signaling { ADDIN EN.CITE <EndNote><Cite><Author>Yang</Author><Year>2002</Year><RecNum>44</RecNum><DisplayText>(Yang et al., 2002b)</DisplayText><record><rec-number>44</rec-number><foreign-keys><key app="EN" db-id="weddavdkawd0dedw5yxpszd9wswf0wzfv">44</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Yang, X. S.</author><author>Dormann, D.</author><author>Munsterberg, A. E.</author><author>Weijer, C. J.</author></authors></contributors><auth-address>Univ Dundee, Wellcome Trust Bioctr, Div Cell & Dev Biol, Dundee DD1 5EH, Scotland</auth-address><titles><title>Cell movement patterns during gastrulation in the chick are controlled by chemotaxis mediated by positive and negative FGF4 and FGF8</title><secondary-title>Developmental Cell</secondary-title><alt-title>Dev Cell</alt-title></titles><pages>425-437</pages><volume>3</volume><number>3</number><keywords><keyword>sex myoblast migration</keyword><keyword>growth-factor receptor</keyword><keyword>avian gastrulation</keyword><keyword>primitive streak</keyword><keyword>convergent extension</keyword><keyword>xenopus gastrulation</keyword><keyword>mouse gastrulation</keyword><keyword>mesoderm movement</keyword><keyword>c-elegans</keyword><keyword>fate map</keyword></keywords><dates><year>2002</year><pub-dates><date>Sep</date></pub-dates></dates><isbn>1534-5807</isbn><accession-num>WOS:000178132800015</accession-num><urls><related-urls><url><Go to ISI>://WOS:000178132800015</url></related-urls></urls><electronic-resource-num>Doi 10.1016/S1534-5807(02)00256-3</electronic-resource-num><language>English</language></record></Cite></EndNote> }. The results show that this significantly increased NCC migration – implying that the FGFR1 plays a crucial role in the

migration process. It has been reported that blocking FGFR1 delayed cardiac NCCs migration (Sato et al., 2011), which is inconsistent with our conclusions. However, it has also been reported that FGF could control the timing of NCC emigration at the trunk level (Martinez-Morales et al., 2011). We agree with the latter and demonstrated that FGF signaling significantly affected NCC migration.

Cell adhesion is a fundamental morphogenetic process that regulates normal embryonic development. Cadherins, a large family of calcium-dependent cell adhesion molecules, are spatiotemporal distribution in the developing embryo and play a crucial role in determining the formation of NCCs. Neural crest cells express different sets of cadherins at different stages of development that includes induction, migration and subsequent differentiation. It has been reported that N-cadherin expression was essential for NCC separation from the neural tube and also allows the NCCs to interact with each other during migration. During NCC induction, E-cadherin expression is down-regulated in the neural plate and replaced by N-cadherin { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. N-cadherin is negatively regulated by the combined actions of BMP signaling and proteolysis { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. NCC migration is a complex process that is directed by the intrinsic properties of NCCs and their interaction with the external environment. There is accumulating evidence that cell-to-cell interaction is also crucial for establishing and maintaining the directionality of NCC migrations { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. It has been reported that increased N-cadherin expression was important for NCC migration { ADDIN EN.CITE { ADDIN EN.CITE.DATA }} and N-cadherin was regulated by FGF signaling pathway { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. In this study, we revealed that blocking FGF signaling by inducing Sprouty2 expression in the neural tube increased NCC migration. Interestingly, we did not find any corresponding changes in N-Cadherin expression in the cranial and trunk neural tube. This implies that the effects of FGF signaling on NCC bioactivities were independent of N-Cadherin. It has been reported that FGF-2 could increase N-cadherin expression in human calvaria osteoblasts, which contradicts our present results { [键入文字]

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ISI>://WOS:000167248700006</url></related-urls></urls><language>English</language></record></Cite></EndNote>}. This contradiction may be attributed due to the differences in species and different cell types.

Cell proliferation at the dorsal side of neural tube is important for NCC development and subsequent migration { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Presently, we have demonstrated that blocking FGF signaling, by inducing Sprouty2 expression in the neural tube, could significantly increase Cyclin D1 expression in the neural tube cells. This suggests that normally FGF signaling inhibits pre-migratory NCC proliferation through MAPK signaling, which explains why blocking FGF signaling in HH10 embryos could significantly increase the number of cranial and trunk NCCs.

Embryo development is a complex and dynamic process and the embryonic environment plays an important role in maintaining normal development { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. FGF are secreted by embryonic cells into this environment and through autocrine or paracrine manner regulates brain, heart, liver and kidney organogenesis { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. It has been reported that FGF8 is expressed in early stage embryos and knocking out this gene severely affects cardiac NNC proliferation and survival. Moreover, FGF8 deficiency in cardiac NCCs would lead to abnormal development of the heart outflow tract { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Similarly, FGF10 is indispensable for normal liver development and knocking out this gene severely affects hepatocyte proliferation and survival, resulting in a small abnormal liver { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. FGF23 is an important regulator of mineral homeostasis which rises sharply in patients with kidney disease. FGF23 could apparently activate fibroblasts and perpetuate the profibrotic signal transduced by the injured kidney - which further increase the progression of the disease { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Hence, FGF signaling plays an important role in embryonic development, homeostasis and disease development.

In conclusion, we have demonstrated that FGF signaling plays an important role in NCC development and migration. At the neurulation stage, blocking FGF signaling [键入文字]

in the developing neural tube increases NCCs migration, which is partly attributed to FGF's ability to modulate pre-migratory NCC proliferation. Nevertheless, there is still a lot that is unknown about how FGF interacts with NCC which remains to be investigated in the future.

Acknowledgements

We would like to thank Prof. Chaya Kalcheim for the Cyclin D1 plasmid. This study was supported by NSFC grant (31401230; 81571436); Science and Technology Planning Project of Guangdong Province (2014A020213008) Science and Technology Program of Guangzhou (201510010073); The Fundamental Research Funds for the Central Universities (21615421). The authors declare that they have no conflict of interest

Competing financial interest

The authors declare that they have no conflict of interest.

References { ADDIN EN.REFLIST }

Figure legends

Figure 1. Induced Sprouty2 expression in chick neural tube increases HNK1⁺ neural crest cells production

Half-side of the neural tube in HH10 chick embryos were transfected with either GFP or Sprouty2-GFP constructs and then allowed to develop for 8 - 10 hours. Immunofluorescent staining to demonstrate HNK1 expression was performed on transverse sections of the transfected embryos (left-hand-side of the neural tube transfected with the constructs while right-hand-side served as the non-transfection control). **A1-D1**: Representative transverse sections of embryos transfected with GFP (A1) or Sprouty2 (B1) at the cranial level, and GFP (C1) or Sprouty2 (D1) at the trunk level. **A2-D2**: Immunofluorescent staining showing HNK1 expression in embryos transfected with GFP (A2) or Sprouty2 (B2) at cranial level, and GFP (C2) or Sprouty2 (D2) at the trunk level. **E**: The bar chart showing the frequency of the

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phenotype following GFP or Sprouty2 transfection. **F**: The bar chart showing the comparison of HNK1⁺ neural crest cell areas between control and transfected side. **A3-D3**: are merge images of A1-D1 and A2-D2, respectively. **E**: Bar chart showing the frequency of phenotypes following GFP or Sprouty2 transfection. **F**: Bar chart comparing areas containing HNK1⁺ NCCs between control and transfected sides. Scale bars = 100μm in A1-B3 and 50μm in C1-D3.

Figure 2. Inhibiting FGFR1 expression in chick neural tube increases HNK1⁺ neural crest cells production

Half-side of the neural tube in HH10 chick embryos were transfected with either GFP or Dn-FGFR1-GFP constructs and then allowed to develop for 8 - 10 hours. Immunofluorescent staining to demonstrate HNK1 expression was performed on transverse sections of the transfected embryos (left-hand-side of the neural tube transfected with the constructs while right-hand-side served as the non-transfection control). **A1-D1**: Representative transverse sections of embryos transfected with GFP (A1) or Dn-FGFR1 (B1) at the cranial level, and GFP (C1) or Dn-FGFR1 (D1) at the trunk level. **A2-D2**: Immunofluorescent staining showing HNK1 expression in embryos transfected with GFP (A2) or Dn-FGFR1 (B2) at cranial level, and GFP (C2) or Dn-FGFR1 (D2) at the trunk level. **A3-D3**: are merge images of A1-D1 and A2-D2, respectively. **E**: Bar chart showing the frequency of phenotypes following GFP or Dn-FGFR1 transfection. **F**: Bar chart comparing areas containing HNK1⁺ NCCs between control and transfected sides. Scale bars = 50μm in A1-D3.

Figure 3. Sprouty2 does not affect N-Cadherin expression in chick neural tube

A1-D1: Representative transverse sections of embryos transfected with GFP (A1) or Sprouty2 (B1) at the cranial level, and GFP (C1) or Sprouty2 (D1) at the trunk level. **A2-D2**: Immunofluorescent staining showing N-Cadherin (N-Cad) expression in embryos transfected with GFP (A2) or Sprouty2 (B2) cranially and GFP (C2) or Sprouty2 (D2) at the trunk. **A3-D3**: are merge images of A1-D1 and A2-D2, respectively. **G** and **E**: Bar charts showing the frequency of phenotypes following GFP
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or Sprouty2 transfection. F: Bar chart comparing N-Cad⁺ areas in neural tubes between control and transfected side. G: RT-qPCR analysis revealed the relative N-Cadherin expression levels in GFP and Sprouty2 transfected tissues. Scale bars = 25μm in A1-D3.

Figure 4. Sprouty2 increased Cyclin D1 expression in chick neural tubes

Half-side of the neural tube in HH10 chick embryos were transfected with either GFP or Sprouty2-GFP, and then incubated further for 8 -10 hours. *In situ* hybridization was performed on the transfected embryos to identify cells expressing Cyclin D1 (left-side transfected; right-side un-transfected control). **A1-D1**: Representative transverse sections of embryos transfected with GFP (A1) or Sprouty2 (B1) at the cranial level, and GFP (C1) or Sprouty2 (D1) at the trunk level. **A2-D2**: *in situ* hybridization showing Cyclin D1 expression in transverse sections of embryos transfected with GFP (A2) or Sprouty2 (B2) at cranial level, and GFP (C2) or Sprouty2 (D2) at the trunk level. **E**: Schematic drawing comparing Cyclin D1 expression in neural tubes between control and transfected side. **F**: Bar chart showing the frequency of the phenotypes following GFP and Sprouty2-GFP transfections. Abbreviation: NT, neural tube. Scale bars = 25μm in A1-D2.

Supplementary Fig 1. Gene transfection of neural tubes in chick embryos

A: Schematic diagram showing how DNA microinjection and electroporation were conducted in the chick embryos. **B-E**: showing GFP expression in the neural tube at cranial (B-C) and trunk (D-E) levels. **B1-E1**: Transverse sections of embryos at levels indicated by dotted lines in B-E respectively. Abbreviation: NT, neural tube; So, somite. Scale bars = 250μm in B-E and 25μm in B1-E1.

Supplementary Fig 2. Induced Sprouty2 expression in chick neural tube increases the production of PAX7⁺ neural crest cells

Half-side of HH10 chick neural tubes were transfected with either GFP or Sprouty2-GFP, and then incubated for 8-10 hours. Immunofluorescent staining to [键入文字]

demonstrate PAX7 expression was performed on transverse sections of the transfected embryos (left-hand-side of the neural tube transfected with the constructs while right-hand-side served as the non-transfection control). **A2-B2:** Immunofluorescent staining showing PAX7 expression in embryos transfected with GFP (A2) or Sprouty2 (B2) cranially. **A3-B3:** The merge images of A1-B1, A2-B2 and DAPI respectively. **C:** Bar chart showing the frequency of phenotypes following GFP or Sprouty2 transfection. **D:** Bar chart comparing areas occupied PAX7⁺ NCCs control and transfected sides. Scale bars = 50 μ m in A1-B3.