Dysregulation of Wnt-Signaling and a Candidate Set of miRNAs Underlie the Effect of Metformin on Neural Crest Cell Development

POULOMI BANERJEE,a SUNIT DUTTAb RAJARSHI Pala

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ABSTRACT

Neural crest cells (NCC) are a population of epithelial cells that arise from the dorsal tube and undergo epithelial-mesenchymal transition (EMT) eventually generating tissues from peripheral nervous system, melanocytes, craniofacial cartilage, and bone. The anti-diabetic drug metformin reportedly inhibits EMT in physiological conditions like cancer and fibrosis. We hypothesize that perturbation of EMT may also contribute to developmental disabilities associated with neural crest (NC) development. To understand the molecular network underlying metformin action during NC formation, we first differentiated murine embryonic stem (ES) cells into NCC and characterized them by demonstrating spatiotemporal regulation of key markers. Metformin treatment prompted a delay in delamination of NCC by inhibiting key markers like Sox-1, Sox-9, HNK-1, and p-75. We then revealed that metformin impedes Wnt axis, a major signaling pathway active during NC formation via DVL-3 inhibition and impairment in nuclear translocation of β-catenin. Concomitantly we identified and tested a candidate set of miRNAs that play a crucial role in NC cell fate determination. Further studies involving loss and gain of function confirmed that NCC specifiers like Sox-1 and Sox-9 are direct targets of miR-200 and miR-145, respectively and that they are essentially modulated by metformin. Our in vitro findings were strongly supported by in vivo studies in zebrafish. Given that metformin is a widely used drug, for the first time we demonstrate that it can induce a delayed onset of developmental EMT during NC formation by interfering with canonical Wnt signaling and miRNA deregulation of miR-145 and miR-200.

INTRODUCTION

The neural crest (NC) is a unique multipotent stem cell population that originates from the neural plate border and epidermal ectoderm during neurulation [1]. Once specified they subsequently start detaching from the neuroepithelium and migrate to distant sites wherein they differentiate into diverse cell types ranging from neurons and glia of sensory, autonomic, and enteric ganglia, to secretory cells of the medulla, melanocytes, smooth muscle cells, bone, and cartilage cells [2]. This developmental process appears to take place in three distinct stages: (a) fate determination of premigratory NC; (b) delamination from the neural epithelium/neural tube; and (c) migration of these cells following distinct routes [3]. Since its commitment, neural crest cells (NCC) undergoes complete and gradual segregation from the neural epithelium through epithelial-mesenchymal transition (EMT) and hence has emerged as a relevant model to understand EMT in “non-malignant environment” [4] (Fig. 1A).

Every step of NCC development is minutely orchestrated by sequential interplay of several...
NC specifier genes such as FoxD3, Sox E group, HNK-1, p-75, Pax-7 as well as bonafide NCC transcription factors like AP2-α, Snail, Sox-5, Sox-9, and Sox-10 [5–7]. Timely activation of signaling pathways like Wnt, bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and retinoic acid [8, 9] is also required for the induction of NC during gastrulation. Sox E group members like Sox-9 and Sox-10 are important players in regulating the effector genes for NCC derivatives; Sox-10 directly regulates MITF during melanocyte development [10]. Moreover, EMT during NC development is tightly controlled by cadherin switch for transition from E-cadherin to N-cadherin followed by N-cadherin to Cadherin-6, where Cadherin-6 marks the premigratory NCC in mouse. In addition, the NCC specifiers mentioned earlier are also reported to control Rho-B expression during migration [11].

Recent epidemiological studies indicate the involvement of various drugs and food supplements to congenital abnormalities and developmental defects with impaired NC formation [12]. According to an earlier report [13] one third of birth defects are associated with NC development. Metformin is one such popular antidiabetic drug often prescribed during pregnancy; but it is shown that metformin can have an inhibitory role during EMT [14]. It is also indicated that metformin can inhibit axonal growth thereby interfering in neurogenesis [15]. These reports prompted us to investigate the mode of metformin action during NCC delamination via EMT. Again there is an increasing body of evidence suggesting a strong connection of microRNAs (miRNA) with neurogenesis and that it is manifold when compared to any other cell systems [16]. Therefore, in this study we also explored the role of a candidate set of miRNAs that are reported to be associated with NC development [17].

With this background, we intended to develop a suitable model system for examining the sequential changes in NC development post metformin treatment. We considered murine embryonic stem cells (ESC) as they are able to differentiate into every cell type of an organism and have been shown to recapitulate in vivo development in a culture dish [18, 19]. Our tailor-made NC differentiation protocol displayed the appropriate spatiotemporal changes in cell morphology across progressive stages of differentiation accompanied with proper interplay of transcription factors and structural markers. We observed that upon treatment with metformin there was impairment in the usual course of NCC specification, delamination as well as migration which was due to abrogation in the Wnt pathway in collaboration with a key set of miRNAs. In parallel, we also conducted in vivo studies to confirm whether metformin produced similar effects in zebrafish embryos or not.

**MATERIALS AND METHODS**

**Maintenance and Differentiation of Mouse ES Cells**

R1 murine ES cell line was grown on inactivated mouse embryonic feeders (Stem Cell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) and gradually adapted to feeder free conditions by culturing on Geltrex (Life Technologies, Rockville, MD, http://www.lifetech.com). Once they reached 60%–70% confluence, undifferentiated ES cells were
induced for NCC differentiation with neural crest induction media (1 × N2 in Neurobasal, 10% albumax, 20 ng/ml FGF2) on laminin (20 µg/ml) (all from Life Technologies)-coated tissue culture dishes (Corning Life Sciences, Acton, MA, http://www.corning.com/lifesciences) for 8 days and then switched to neural crest differentiation media (same as earlier but supplemented with 10 ng/ml BMP4) for 2–3 days. These NCC were subcultured till passage (P)-5 routinely using Accutase (Life Technologies) on laminin-coated dishes. For Schwaan cells and smooth muscle cell differentiation, we followed the protocol reported by Aihara et al. [20]; whereas studies on chondrogenic and adipogenic differentiation were conducted using respective StemPro differentiation kits (Life Technologies).

Drug Treatment
Metformin (Sigma) was added to the differentiation media in 1 mM concentration; 10 ng/ml Wnt3A (Peprotech, Rocky Hill, NJ, http://www.peprotech.com) and 25 ng/ml CHIR99021 (Sigma) was added to the same cocktail for the rescue and validation experiments, respectively. Different concentrations of metformin—1 mM, 1.5 mM, 3 mM, and 6 mM were tested during NCC differentiation in order to optimize the appropriate/effective dose for further studies (Supporting Information Fig. S1A–S1F). Cell viability was evaluated postdrug treatment using 7-Aminoactinomycin D (7AAD) (Sigma Aldrich, St. Louis, http://www.sigmaaldrich.com) on a flow cytometer, and histograms were prepared (Supporting Information Fig. S1G–S1I).

Gene Expression Studies
For quantitative polymerase chain reaction (Q-PCR) analysis, RNA was extracted by RNeasy kit (Qiagen, Hilden, Germany, http://www1.qiagen.com), and cDNA conversion was done using RevertAid kit (Thermo Scientific) as per the manufacturer’s instructions (https://tools.thermofisher.com/content/sfs/manuals/MAN0012955_RevertAid_RT_UG.pdf). miRNA was isolated with miRVANA (Life Technologies) kit following the manufacturer’s protocol. 2 µg of RNA and miRNA was used for cDNA conversion. Q-PCR was done with SYBER Green master mix (Life Technologies) using mRNA/miRNA-specific primers (Supporting Information Table SII, SIII) on StepOnePlus (Life Technologies) and was analyzed by StepOne software (version 2.2.2). Normalization was performed based on the average of expression of constitutive gene GAPDH.

Immunocytochemistry
For indirect immunofluorescence, cells were harvested at appropriate time points and fixed in 2% paraformaldehyde for 20 minutes at room temperature (RT) followed by washing in 1 × PBS. Cells were then permeabilized for 5 minutes with 0.1% Triton X-100 (Sigma), then blocked for 1 hour at RT with 4% fetal bovine serum (FBS), and incubated with primary antibodies (Supporting Information Table SIII). Followed by overnight primary antibody incubation at 4°C, cells were washed with 1 × PBS twice and probed with secondary antibodies for 1 hour at RT. After secondary antibody treatment cells were washed thoroughly with 1 × PBS and observed under fluorescent microscope (Olympus IX73). For antibodies specific against membrane epitope cells were not permeabilized.

Immunoblotting and Coimmunoprecipitation
For immunoblotting, cells were lysed using a cell lysis buffer from Cell Signaling Technology (www.cellsignal.com) (Cat no: 9803) according to the manufacturer’s instructions along with a protease inhibitor (Sigma) and phosphatase inhibitor (Sigma) for the detection of DVL-3 (DSHB, Iowa City, IA, http://www.uiowa.edu/~shbwww), E-cadherin (BD), β-catenin (BD), TCF4 (DSHB), and β-actin (BD). For membrane protein isolation a specialized kit from Abcam (Cambridge, U.K., http://www.abcam.com; ab65400) was used. Antibodies for immunoprecipitation were added to Sepharose-G beads (Life Technologies) and incubated for 4 hours at 4°C followed by binding under rotary agitation with 100 µg of cell lysates at 4°C overnight. Immune complexes along with the beads were collected after three washes with TBST for 15 minutes each. These immune precipitates were then eluted from the beads by heating samples in a loading buffer with SDS as denaturant. The eluted samples were then subjected to immunoblotting and detected by the antibody specific for its respective interacting partner by following standard procedures. The proteins were detected using a Chemiluminescence kit (Millipore Billerica, MA, http://www.millipore.com).

Flow Cytometry
For flow cytometry analysis cells were detached using Accutase and stained for 1.5 hour with primary antibody p-75 (Millipore) and with 7AAD (Sigma) for 10 minutes at RT to check for cell viability. After incubation with primary antibody, they were washed twice with 1 × PBS and probed with appropriate secondary antibody for 45 minutes at RT. Cells were washed once with 1 × PBS and run in FACS Caliber (BD). Analysis was done using CellQuest pro software.

Transfection and Luciferase Assay
miRNA inhibitors and mimics were procured from Life Technologies and were transfected in stage-1 differentiated cells using Lipofectamine 2000 (Life Technologies) following the manufacturer’s protocol. RNA pellets were collected after 36 hours post-transfection. 3'UTR-Luc constructs for Sox-1 and Sox-9 were purchased from GeneCopoeia (http://www.genecopoeia.com/) and were cotransfected with mimics and inhibitors of respective miRNA independently with scrambled-GFP (green fluorescent protein) as transfection control. pMIR-Glow (Promega) cloned with a 3'UTR of a nonspecific target integrin was used for luciferase control; luciferase activity was measured on TECAN microplate reader.

In Vitro Scratch Assay
A confluent monolayer of NCC were grown in passages and scratched with 200 µl tip as described by Zimmer et al. [21] in migration assay of NC cells (MINC) assay in presence of dimethyl sulfoxide (DMSO) (vehicle control). They were monitored by bright field microscope, and the images were captured at regular intervals across 36 hours. The percentage of healing was calculated with respect to the difference of distance between scratched margins using Image J, and a graph was plotted in Microsoft Excel.
Zebrfish Maintenance and In Situ Hybridization

Zebrfish (Danio rerio) embryos from wild-type and Tg (−4.9 sox10:eGFP) transgenic line [22] were obtained by natural crosses and staged according to published literature [23]. Embryos were incubated at 28.5°C with different doses (10 mM, 15 mM, 25 mM, and 50 mM) of metformin (Sigma) in embryo medium. Metformin was removed by washing several times in embryo medium and incubated in embryo medium without metformin. Whole mount in situ hybridization (ISH) were performed as previously described [24] using antisense Digoxigenin probes for snai2, sox9b, mitfa, emx3, krox20, and myod. Images were taken with Leica M205 microscope.

Statistical Analysis

Results are represented as mean ± SD with experimental replicates indicated in the figure legends. Statistical significance was calculated using the Student’s t test. p ≤ 0.05 was accepted as statistically significant.

RESULTS

Differentiation of NCC from ES Cells

In order to study NC development in vitro, we developed a simple two-step protocol for NC differentiation from feeder-free murine ES cells and then authenticated the spatiotemporal expression of NCC-specific markers during progressive stages of NC differentiation (Fig. 1A). Visible change in morphology from flattened neuroepithelial cells to elongated irregular mature cells (Fig. 1B–1E) was observed within 8 days of FGF-2 treatment. Q-PCR analysis displayed enhanced expression of key neuroprogenitor markers such as Mushashi-1, Sox-9, FoxD3, HNK-1, and Snail (Fig. 1H–1N). More precisely, at stage 1 (day 1–3 of differentiation) there was an enrichment of neuroepithelial and premigratory cell population marked by increase in Pax-7, Sox-1, HNK-1, Nestin followed by stage 2 (day 4–8 of differentiation) characterized by concomitant decline in Pax-7/HNK-1 and contrasting increase in typical NCC markers such as FoxD3, AP2-α, and p-75 (Fig. 1I–1N). We further confirmed the multilineage differentiation potential of these naïve NCCs by guiding them to form schwann cells, smooth muscle cells, chondrocytes, and adipocytes (Fig. 1O–1T). Subsequently, we were able to maintain these NC cells till passage number 5 (P-5) without compromising their basic characteristics; wherein expression of typical NCC markers like AP2-α, p-75, and Sox-9 were consistent across P-2 and P-5 (Fig. 1F, 1G, 1U–1W, 1S–1J, 1K).

Normal Course of NCC Differentiation is Impaired by Metformin Treatment

Phase contrast microscopy and Q-PCR analysis revealed that 1 mM metformin treatment impairs the normal course of NC differentiation. Transition of neuroepithelial like cells to elongated mesenchymal like phenotype followed by characteristic EMT-like scatter was not visible in metformin-treated cells (Fig. 2A–2C). This was accompanied by deregulation of neuroectoderm and premigratory markers like Pax-7, Sox-1, HNK-1, and FoxD3 (Fig. 2D). Downregulation of NCC markers like p-75 and Ap2-α during differentiation stage 2 (Fig. 2D) indicated that the usual course of NC differentiation post-treatment is delayed or deferred. Further flow cytometry demonstrated significant reduction of p-75 (+) ve population in the treated set at stage 2 (Fig. 2Ei, ii). The above data suggests that metformin hampers the sequential expression of markers during NC delamination and migration.

Metformin Causes Improper Synchronization of NC-Specific Proteins

Given that metformin causes a delay in NCC formation, we now designed an experiment to systematically study the kinetics of metformin action. E-cadherin represents non-neural ectoderm [25]; while Sox-1, Sox-9, and Slug mark the cells prone to delamination [26, 27]. During NC induction, E-cadherin expression gradually weakens and gets replaced by N-cadherin and cadherin-6, and it is typical of EMT. Likewise, our data revealed an overlap of E-cadherin and Sox-9 in both control and treated cultures at day 2 (Fig. 2F–2K). In contrast after day 4 we witnessed gradual loss of E-cadherin and gain in Sox-9 compared to the treated cultures that displayed an unexpected persistence of E-cadherin expression (Fig. 2L–2Q). Furthermore, on day 8 there was a complete loss of E-cadherin with a prominent gain in Sox-9 in the control; whereas the treated cells had strong expression of E-cadherin and much less of Sox-9 (Fig. 2R–2W). These findings suggested that metformin facilitates prolonged expression of E-cadherin and thereby causing delayed delamination of NCC that was further supported by gene expression data (Fig. 2X). Spatiotemporal enrichment of N-cadherin and cadherin-6 expression was also observed in the control but not in the treated cells during both stages of differentiation (Fig. 2Y, 2Z).

Next, in order to rule out nonspecific effect of metformin in NC differentiation, we performed colocalization studies of Slug (early NCC marker) and SSEA-1 (undifferentiated mouse ESC marker). Our data indicated that metformin failed to produce any effect on the pluripotency of ESCs as evidenced by gradual decline in the expression of SSEA-1 across days 2, 5, and 8 in differentiation both in control and treated cultures (Fig. 3A–3R). However, Slug expression was considerably decreased as a result of metformin (Fig. 3A–3R). These data were consistent with our Q-PCR results wherein gain in Slug expression with time was abridged in treated set when compared to control; no change in Nanog and Oct-4 levels further strengthened this observation (Fig. 3S, 3T). Other important markers for NC development like p-75, HNK-1, and Sox-1 also declined in the treated sets when compared to control (Fig. 3U–3X; Supporting Information Fig. S2A–S2N). Taken together these data indicate a temporal retardation in NCC specification between days 2–8 in the metformin-treated cultures that might be attributed to the enhanced manifestation of E-cadherin that could interrupt the onset of EMT during NC delamination.

Metformin Hinders the Migration Rate of In Vitro Generated NCCs by Regulating MMP-2 and Rho Kinase

To assess the migratory capacity of NCC after metformin treatment, cells were seeded and grown as a monolayer on laminin-coated dishes and scratched for MINC assay as described by Zimmer et al [21]. The potential of cell migration was assayed in terms of cells filling the “cell-free scratch area.” Figure 4A–4F, Supporting Information Figure S3A, S3B
clearly showed that metformin-treated cells underwent a delayed rate of gap filling. We also found considerable reduction in the expression of migration related molecules like MMP-2, MMP-9, and Rho-B (Fig. 4G–4I) and that could be ascribed to the slower rate of migration. Conversely, both of these markers are direct/indirect targets of canonical Wnt signaling.

Metformin Perturbs Canonical Wnt Axis

One of the important pathways during NCC formation is Wnt and many of its targets like Sox-9, Sox-2, ZEB2, Slug, Snail, and Pax-7 play a crucial role in NC development and EMT with respect to both specification and differentiation (Fig. 4J). Once we figured that the principle players of canonical Wnt pathway are deregulated, we went on to explore the status of β-catenin. Figure 5A reveals that in the untreated cells β-catenin is present in the nucleus at day 2, but in the treated cells, β-catenin was primarily localized in the membrane (Fig. 5B). As we analyzed the day 5 cultures we could see treated cells bearing nuclear β-catenin was much reduced when compared to untreated samples (Fig. 5C, 5D). Again, immunoblotting results clearly indicated that there is a significant reduction in DVL-3 expression in the metformin-treated sample (Fig. 5E). At the same time we observed a significant downregulation in the levels of Wnt mediators like DVL-3, LEF-1 and upregulation in Groucho expression in the metformin-treated set (Fig. 5F–5H). Therefore, we inferred that metformin could impede with canonical Wnt axis by inhibiting DVL-3 and resulting in impairment of β-catenin translocation to the nucleus. Moreover coimmunoprecipitation (Co-IP) data with β-catenin and TCF4 further supported this impairment in nuclear localization of β-catenin (Fig. 5I, 5J).

Disruption of E-Cadherin Switch Delays the Onset of EMT During NCC Specification

Concomitant with Wnt pathway modulation and impairment in β-catenin translocation, we detected that E-cadherin localization is confined to the membrane in the treated samples but abolishes with time in the control (Fig. 5K). Loss of E-cadherin expression is extremely crucial for β-catenin translocation in the nucleus [30]. Furthermore, it is shown that constitutive expression of E-cadherin in membrane impairs
invasiveness and leads to an arrest of EMT. Hence, we performed Co-IP for the membranous fraction of cells against $\beta$-catenin and E-cadherin complex; E-cadherin was found in the same complex with $\beta$-catenin in the treated samples, while the coexistence was much less in control (Fig. 5L, 5M). Earlier Q-PCR not only showed much higher expression of E-cadherin but also significant downregulation of E-cadherin repressors like Snail and ZEB-1 in the treated cells (Fig. 2K, 2L). Taken together metformin appears to deregulate canonical Wnt axis by directly inhibiting DVL-3 arresting the nuclear translocation of $\beta$-catenin which in turn fails to bind to TCF-4, thus deactivating its respective target promoters like FoxD3 and Slug leading to increased expression of E-cadherin.

Identification of miRNA(s) Unique to Metformin Induced Perturbation in NC Specification

In order to validate the involvement of Wnt signaling, we designed a rescue experiment by adding exogenous Wnt3A in the metformin-treated cultures with an anticipation of a possible recovery with appropriate control (Supporting Information Fig. S4). Figure 5G, 5H demonstrated a twofold rise in DVL-3 and Groucho levels upon addition of Wnt 3A. Similarly, the expression of key genes crucial for NCC specification like HNK-1, Ap2-$\alpha$, FoxD3, Sox-2, p-75, Nestin, and Rho-B (Fig. 6A), which are potential targets for Wnt pathway, also increased. Surprisingly, ZEB-1, MMP-9, cdc-42, and Sox-9 did not change much after Wnt3A addition (Fig. 6B). Furthermore, to verify that metformin acts through Wnt pathway, we treated the cells with intracellular Wnt/GSK-3 $\beta$ inhibitor (CHIR 99021). We demonstrated a significant downregulation of NCC markers (Sox-1, p-75, Sox-9) and Wnt targets (LEF-1, DVL-3, Snail, MMP-9) in stage 1 for CHIR + metformin-treated groups compared to untreated and only CHIR-treated groups (Supporting Information Fig. S5). Taken together, these experiments suggested that besides deregulating canonical Wnt axis metformin also modulates NCC specifiers by parallel mechanisms.

Since miRNAs are closely associated with neurogenesis [29], we sought to explore whether metformin also modulates the “NC miRNA signature” and alters the normal course of NCC commitment. We used Bioinformatics’ tools including ingenuity pathway analysis, TargetScan, and miRanda to screen for NCC-specific target genes with respect to miRNAs.

Figure 3. Effect of metformin on fate commitment of ES cells. (A–R) shows the images of SSEA-1 costained with Slug across days 2, 5, and 8 in differentiation. All scale bars = 100 $\mu$m; all data are representative of at least three independent experiments. (S, T): Represents Nanog and Oct-4 expression in treated and untreated cells across stages 1 and 2, respectively. All quantitative polymerase chain reaction values are normalized to GAPDH and compared to undifferentiated murine ES (mES) cells. (U, W): shows the reduction in expression of HNK-1 in metformin-treated cells. (V, X): Shows Phalloidin which marks the actin fibers during active migration in premigratory neural crest cell; Insets are $\times$ 100 images depicting the localization pattern of Phalloidin and HNK-1. Dotted lines mark the edge of differentiating colonies. Abbreviation: DAPI, 4',6-diamidino-2-phenylindole.
Figure 4. Metformin hinders the migratory ability of neural crest cells. NCC at P-2 was considered for scratch assay. Cells were grown in the form of monolayer and scratch assay was done as described in Materials and Methods section. (A–F): Represents photomicrographs at 0 hour, 12 hours, and 36 hours postscratch in control and metformin-treated sets; the margin of the wound is shown by dotted lines. Scale bars = 100 μm. (G–I): Shows the relative expression of important proteins associated with migration like Rho-B, MMP-2, and MMP-9 in both the stages of NCC differentiation across control and treated sets. Data represented here are considered in triplicates, and SD is calculated from three independent experiments. The relative expression of a gene is represented as fold change between the undifferentiated ES cells to its respective treated and control sets. Significance levels of the values are within $p < 0.01$, and all data are representative of at least three independent experiments. (J): Represents the network overlay of NCC determinants and Wnt effectors constructed using GeneMANIA prediction server (www.genemania.org). Abbreviations: MMP, Matrix metalloproteinases; NCC, neural crest cell.

Figure 5. Metformin impedes WNT axis. (A–D): Represents immunostaining of β-catenin at days 2 and 4 of differentiation across treated and control cells. Star and yellow highlighter indicates the colocalization of β-catenin and nuclear DAPI; scale bars = 100 μm. (E): Shows immunoblotting of DVL-3 protein with β-actin as control lysate of treated and untreated samples at differentiation stage 1. (F–H): Depicts the relative expression levels of LEF-1, Groucho, DVL-3. (I): TCF-4 antibody was immobilized on sepharose-G bead and followed by incubation with nuclear cell lysate of from stage-1 differentiation for crosslinking; TCF-4 interacting partners were then eluted out by boiling in Lamelli buffer and subjected to immunoblotting to detect the presence of β-catenin. Lysates of both controls and treated were checked for the presence of total TCF-4 in the total cell lysates. (J) Shows the densitometric evaluation of the blots for coimmunoprecipitation (co-IP) of nuclear extracts of β-catenin and TCF-4. (K): Represents E-cadherin immunolocalization at days 2 and 4 across treated and untreated cultures, and arrowheads indicate membranous localization; scale bars = 100 μm. (L): Co-IP of membranous β-catenin and E-cadherin was carried out; β-catenin in the total cell lysate was also checked in both control and treated samples. (M): Represents the densitometric evaluation of the blots for co-IP of membranous extracts of β-catenin and E-cadherin. All data are representative of at least three independent experiments. Abbreviation: DAPI, 4',6-diamidino-2-phenylindole.
Figure 6. Metformin deregulates a candidate set of miRNA(s) during NCC formation. (A, B): Depicts the relative expression levels of HNK-1, Ap2-α, FoxD3, Sox-2, p-75, Nestin, Rho-B, Snail, ZEB, MMP-9, cdc-42, N-cadherin, E-cadherin, and Sox-9 upon endogenous Wnt3A addition at differentiation stage-1 across control and treated sets. (C): IPA depicts how Wnt pathway in collaboration with a battery of miRNAs can modulate important NCC determinants like cdc-42, CDH-1, CDH-2, Pax-3/7, MITF, Twist, ZEB, and so on. (D): Represents the heat map characterizing the deregulation of miRNAs in metformin-treated cells versus control cells across both the stages of differentiation. Red color shows the minimal expression and green corresponds to the maximum expression. The values for the fold changes of miRNA are summarized in Supporting Information Table SV. The fold changes in treated and control are calculated relative to the undifferentiated ES cells. All the data are representative of three independent experiments. (E): Shows the clustering of miRNAs with regard to the expression across stages 1 and 2 in the treated cells when compared to the control cells. (F, H): Shows relative expression of miR-145, miR-200c compared to stage 1 control, post-transfection with respective mimics and inhibitors at stage 1 of NCC differentiation whereas (G, I) shows the relative expression of Sox-9, and Sox-1 compared to stage 1 samples, post-transfection of stage 1 control cells with miR-145/miR-200c mimics and inhibitors and scrambled, respectively. (J): Represents the 3’-UTR region of Sox-1 and Sox-9 and their seed sequence complementarity with miR-145 and miR-200c, respectively designed for luciferase assay. (K, L): Represents the luciferase reporter analysis of Sox-9 and Sox-1 UTR in HEK293T cells upon binding with miR-145c, miR-200c mimics, and inhibitors respectively. Reporter constructs of pMIR-Glow with 3’-UTR constructs of integrin was used as a positive control. The results are ± SEM of three independent experiments. (M): Represents the proposed schematic model illustrating the mode of metformin action during NCC differentiation. Essentially it shows that metformin treatment impedes canonical Wnt cascade by inhibiting DVL-3 which potentially downregulates E-cadherin repressors thus allowing the unusually persistent expression of E-cadherin. Intracellular recruitment of β-catenin and E-cadherin in turn hampers the former's translocation from membrane to nucleus leading to interruption in the transcription of Wnt targets, thus delaying the onset of EMT. Additionally, our results indicate that metformin also modulates miR-145 and miR-200c via Sox-9 and Sox-1 that are important specifiers of NC development. Abbreviation: UTR, untranslated region.
and identified a candidate set of miRNAs along with their predicted/known targets (Fig. 6C; Supporting Information Table SIV). Based on their expression profile (Fig. 6D; Supporting Information Table SV), we clustered the miRNAs with regard to their expression kinetics in stages 1/2 of NCC differentiation in comparison to control. Cluster 1 (+ –) is characterized by an increase in expression of miRNAs in both stage 1 and stage 2; cluster 2 (+ –) represents those miRNAs that are expressed more in stage 1 than stage 2; cluster 3 (– +) comprises miRNAs that decrease in stage 1 but then gradually increase in stage 2; and cluster 4 (– –) consists of miRNAs which remained unaltered or did not express in any stage of differentiation. Supporting Information Table SV and Figure 6E reveals that expression of most of the miRNAs is deregulated beyond twofold across both stages of differentiation in the metformin-treated cells unlike the controls. To further narrow down to specific miRNA(s) modulated by metformin in defined physiological condition of differentiation, we compared their respective profiles and found that miR-200c and miR-145 underwent maximum, that is, more than 5–10-fold upregulation upon drug treatment across both the stages. Interestingly, this finding fully complies with one previous study which showed that metformin upregulates miR-200 (200a/200b/200c) series and that miR-200 series negatively regulates neural differentiation [30]. Prediction analysis by established software allowed us to screen and identify Sox-1 and Sox-9, both crucial NCC specifiers as novel targets of miR-200 and miR-145, respectively by matching respective seed sequences. Figure 6F–6I demonstrates that upon independent transcription of inhibitors and mimics of miR-145 and miR-200 there is a noteworthy upregulation and downregulation their respective target like Sox-9 and Sox-1. To verify the interaction of respective miRNA and their predicted targets, we considered 3’-UTR luciferase constructs of Sox-9 and Sox-1 (Fig. 6J) and cotransfected them with mimics and inhibitors of respective miRNAs such as miR-200 and miR-145. Our results show (Fig. 6K, 6L) that overexpression of miRNA-145 and –200c decreased the luciferase activities of Sox-9 and Sox-1, respectively. On the other hand combined transfection of 3’-UTR construct, mimics, and respective inhibitors can lead to increased luciferase activity when compared to mimics alone. Taken together, our results confirm that metformin modulates miR-200 and miR-145 which directly target Sox-1 and Sox-9, the chief NCC determinants.

So far, our data suggest that metformin deregulates canonical Wnt axis which potentially downregulates E-cadherin repressors thus allowing the unusually persistent expression of E-cadherin. Intracellular recruitment of β-catenin and E-cadherin in turn harms the former’s translocation from membrane to nucleus leading to interruption in the transcription of Wnt targets, thus delaying the onset of EMT. Additionally, our results indicate that metformin also modulates miR-145 and miR-200 via Sox-9 and Sox-1 that are important specifiers of NC development.

**Metformin Treatment Inhibits NC Development in Zebrafish**

In order to determine the optimal concentration of the drug we incubated zebrafish embryos in various concentrations of metformin in embryo water starting at 2-cell stage and 1,000-cell stage and monitored the development at 15-somite stage. Metformin treatment from two-cell stage was toxic. None of the embryos survived and similarly 50 mM metformin treatment starting from 1,000-cell stage resulted in very low survival (1%–3%) and delayed development of the embryos. However, embryos incubated in 25 mM, 15 mM, and 10 mM metformin starting from 1000-cell stage showed very good survival rate (90%–95%) and normal development similar to control embryos (Supporting Information Fig. S7 and Table SVII). To analyze the expression of NC markers in metformin-treated embryos, we performed the ISH with zebrafish snai2 (slug), and sox9b at 5-somite stage, and observed that expression of snai2 (Fig. 7A, 7B) and sox9b (Fig. 7C, 7D) were reduced in NC precursors (Supporting Information Table SVI). Similarly, metformin treatment leads to loss of reporter gene expression in (sox10:eGFP) transgenic embryos compared to control embryos (Fig. 7E, 7F). Pigment cells are derived from NC and mitfa is a melanocyte marker. To check the effect of metformin in NC differentiation, we have tested the expression of mitfa and skin pigmentation at later stages of development and observed that mitfa expression at 24 hours (Fig. 7I, 7J) and skin pigmentation (Fig. 7G, 7H) were subsequently reduced in metformin-treated embryos as compared to control. To rule out the non-specific effect of metformin in embryo development, we checked the expression pattern of emx3, krox20, and myod in both metformin-treated embryos and control embryos. Similar expression pattern of the markers (Fig. 7K, 7L) was found suggesting the observed defects were NC specific. Together, our data indicate that metformin treatment inhibits specification, migration and differentiation of NC cells during embryo development in zebrafish.

**Discussion**

There is mounting evidence to suggest that any abnormality in the normal course of development may result in several pathologically relevant congenital diseases [13], however this field of developmental neurotoxicity has been less studied. Few reports using in vitro models [21] have elegantly demonstrated how known toxicants like methyl mercury, valproic acid, and lead acetate affect neural precursors. The dynamics of the expression of cadherin-6 was shown by Clay et al. [31] highlighting its importance in regulating EMT—one of the principle phenomena during NCC development. In parallel, it is elucidated that heterogeneous existence of neural and non-neural ectoderm and their ability to undergo EMT during the initial stages of NCC fate determination [27]. On the other hand, it has been well-established that ES cells can serve as an appropriate model to study early embryogenesis [32]. Therefore, we choose the ES cell-based differentiation model with anticipation that phenotypic transition accompanied with a dynamic change in molecular signature of neuroepithelial cells via EMT may be a suitable system to study NC development. Our model depicted that any alterations in mRNA/protein level may allow one to draw a correlation with the abrogated mode of NCC development.

Our study not only deals with development of an in vitro platform to evaluate developmental neurotoxicity but we also
analyzed the time and dose dependent effects of one of the widely administered drug, metformin. There are several reports that upon prolonged treatment with metformin patients suffered from problems related to peripheral neuropathy [33, 34] and furthermore, Cufy et al. [14] also studied its role in inhibiting EMT. As a result, we were tempted to understand the role of metformin in modulating EMT during NC development.

Zebrafish have been used as an attractive model system to study NC development and to understand the genetic basis of human NC disorders, known as neurocristopathies [35–39]. In the last decade, zebrafish have emerged as an excellent tool for phenotype based small molecule screen, for example, zebrafish screens have been successfully used to identify effects of nicotine on spinal motoneuron development [40]. The zebrafish model has also been used for developmental neurotoxicity studies to define the effects of arsenic, methylmercury, and lead [41–43]. Therefore, we used zebrafish as an in vivo model to corroborate the effects of metformin seen in vitro. We confirmed that metformin treatment inhibits induction, migration, and differentiation of NC cells during early embryonic development leading to impairment in melanocyte development at a later stage.

It has been shown that Sip1 modulates EMT in zebrafish during the formation of premigratory cells from neuroepithelium and the absence of sip1 expression leads to aberrant expression of E-cadherin thus hampering the usual onset of EMT [4]. In similar lines, we witnessed normal expression of E-cadherin at the onset of differentiation that marks a non-neuroepithelial population. As we anticipated, in the metformin-treated set, there was neither any downregulation of E-cadherin nor upregulation of N-cadherin followed by Cadherin-6. This finding was supported by a slower rate of NCC migration in the scratch assay and could be attributed to the decreased expression of MMPs, CDC-42, and Rho-B. It is clearly implicated that there is an abrogation in the cues required for NCC specification in the initial stages of differentiation that resulted in impaired migration both in vitro and in vivo.

There is strong evidence proposing that key NCC fate determinants like FoxD3, Slug, and Sox-9 appear to be direct or indirect targets of Wnt signaling cascade and that Wnt plays a major role during NCC development across all species [44–46]. Moreover overexpression of any player in the canonical Wnt signaling pathway such as TCF/β-catenin/Frizzled or ligands like Wnt3A in the whole embryo can facilitate formation of NCC [47]. In addition β-catenin ablation in mouse leads to impaired NCC development. Conversely the effect of deregulation of an important element in Wnt cascade such as DVL is homolog and species dependent [48]. Our study revealed that metformin inhibits DVL-3 and thereby interferes with the normal function of Wnt canonical cascade. It may be one of the contributing reasons for the reduced expression of some of the Wnt targets like Sox-9, Slug, and FoxD3. Furthermore, it was shown that recruitment of E-cadherin to β-catenin in the membrane prevents the nuclear translocation of the later thus affecting the activation of Wnt effector—LEF/TCF complex [28]. Similarly, our data suggests that metformin treatment hampers β-catenin translocation to nucleus, and Co-IP studies confirmed that indeed the aberrant recruitment of β-catenin by E-cadherin is instrumental in the inactivation of the canonical Wnt signaling axis. Intriguingly, when we exogenously added Wnt3A ligand, although we could detect reduction in the expression of key NCC markers and Wnt effectors like p-75, Nestin, FoxD3, Rho-B, DVL-3, and Groucho, the rescue was not as efficient as in the

Figure 7. Metformin inhibits NC development in zebrafish. Control embryos (no metformin treatment, A, C, I, K), embryo with 25 mM metformin treatment (B, D, L), and embryos with 15 mM metformin treatment (J) were fixed at stages indicated in lower left. Expression of snai2 (A, B), sox9b (C, D), mitfa (I, J), and coexpression of emx3, krox20, and myod (K, L) were analyzed by in situ hybridization. Embryos from sox10:eGFP transgenic line were analyzed for reporter gene expression at 15-somite stage with 25 mM metformin treatment (F, F inset) and no treatment (E, E inset). Skin pigmentation at 120 hours in control embryos (G) and in embryos treated with 15 mM metformin (H, as indicated). Scale bar = 100 μm, (A–F) dorsal views, anterior to top, (G–L) lateral views anterior to the left; s, somite; ov, otic vesicle. All data are representative of at least three independent experiments.

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case of Sox-9, N-cadherin, cdc-42, E-cadherin, Snail, ZEB-1, and MMPs. We validated our results with a specific Wnt/β-catenin pathway inhibitor. Therefore, we hypothesized that perhaps Wnt pathway is not the only target of metformin and there could be some other parallel mechanism underpinning metformin action. Eventually, we found that some of these NCC responsive genes are common targets of both miRNAs as well as Wnt axis [17]. Our Q-PCR data demonstrated a severe misregulation of several miRNAs involved during NCC formation, and based on the level of expression we identified miR-145, miR-200, and their respective novel targets. It has been reported that metformin can inhibit the key processes of EMT like invasion and migration by deregulating miR-200 series [49]. Moreover, miR-200 family (200a/200b/200c) has been reported to regulate of several miRNAs involved during NCC formation, and could be some other parallel mechanism underpinning metformin induced impairment in the activation of canonical Wnt axis which leads to an inappropriate cadherin switch. Metformin impairs canonical Wnt axis by inhibiting DVL-3 and the sustained expression of E-cadherin promotes a stable interaction with membrane bound β-catenin thus impairing its nuclear translation. As a result there is a curtailed activation of Wnt targets: Pax-7, Sox-9, and FoxD3 in concert with miR-145 and miR-200c, which finally results in the improper induction of NCC. Our in vivo data in Zebrafish provides supportive evidence that skewed differentiation and migration of NCC may lead to defective crest development. It is known that Frontonasal dysplasia, DiGeorge syndrome, and many other diseases [60] are associated with abnormal NC development. Therefore, our study has significant therapeutic importance and it raises important questions about the associated side effects of this widely popular drug.

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AUTHOR CONTRIBUTIONS

P.B.: design, data analysis and interpretation, collection and assembly of data, and manuscript writing; S.D.: design, data analysis and interpretation, and manuscript writing; R.P.: conception and design, data analysis and interpretation, final approval of the manuscript, and financial support.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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