Glucose-6-phosphate dehydrogenase is indispensable in embryonic development by modulation of epithelial-mesenchymal transition via the NOX/Smad3/miR-200b axis

Yi-Hsuan Wu¹,², Ying-Hsuan Lee², Hung-Yu Shih³, Shih-Hsiang Chen⁴, Yi-Chuan Cheng³,⁵ and Daniel Tsun-Yee Chiu¹,²,⁶

Abstract
Glucose-6-phosphate dehydrogenase (G6PD) is a housekeeping enzyme involved in the pentose phosphate shunt for producing nicotinamide adenine dinucleotide phosphate (NADPH). Severe G6PD deficiency leads to embryonic lethality, but the underlying mechanism is unclear. In the current study, the effects of G6PD on epithelial–mesenchymal transition (EMT), especially during embryonic development, were investigated. The knockdown of G6PD induced morphological changes, accompanied by the suppression of epithelial markers, E-cadherin and β-catenin, in A549 and MDCK cells. Such modulation of EMT was corroborated by the enhancement of migration ability in G6PD-knockdown A549 cells. Zebrafish embryos with g6pd knockdown exhibited downregulation of the E-cadherin/β-catenin adhesion molecules and impaired embryonic development through reduction in epiboly rate and increase in cell shedding at the embryo surface. The dysregulation in zebrafish embryonic development caused by g6pd knockdown could be rescued through human G6PD or CDH1 (E-cadherin gene) cRNA coinjection. The Smad3/miR-200b axis was dysregulated upon G6PD knockdown, and the reconstitution of SMAD3 in G6PD-knockdown A549 cells restored the expression of E-cadherin/β-catenin. The inhibition of NADPH oxidase (NOX) activation through the loss of p22phox signaling was involved in the dysregulation of the Smad3/miR-200b axis upon G6PD knockdown. The reconstitution of G6PD led to the recovery of the regulation of NOX/Smad3/miR-200b signaling and increased the expression of E-cadherin/β-catenin in G6PD-knockdown cells. Thus, these results suggest that in the EMT process, G6PD plays an important regulatory role as an integral component of the NOX/Smad3/miR-200b axis.

Introduction
Glucose-6-phosphate dehydrogenase (G6PD) is a housekeeping enzyme with the major function of regenerating nicotinamide adenine dinucleotide phosphate (NADPH) to maintain cellular redox homeostasis¹-⁴. Because NADPH is essential for NADPH oxidase (NOX) and nitric oxide synthase to produce reactive oxygen and nitrogen species for signaling, many new cellular roles of G6PD have been identified. G6PD modulates xenobiotic metabolism via the Nrf2 signaling pathway and affects the xenobiotic metabolizing enzyme expression⁵. Moreover, it acts as a key regulator of the cellular inflammatory and immune response against viral infection⁶,⁷. Because G6PD is a rate-limiting enzyme in the pentose-phosphate shunt, it is essential for regulating energy consumption and glucose...
metabolism\textsuperscript{5}. These studies have indicated that G6PD plays an important role in modulating many cellular functions.

Severe G6PD deficiency is embryonically lethal\textsuperscript{9,10}. G6PD is essential in the regulation of embryonic development. Severe G6PD deficiency in mice causes the demise of male embryos in hemizygous G6PD deficiency and female embryos in heterozygous G6PD deficiency\textsuperscript{7}. Reduced litter size is also observed in mice with slight G6PD deficiency\textsuperscript{11}. The study of a severe G6PD deficiency mouse model delineates that the initial development of all embryos, especially in early gestation, is unimpaired\textsuperscript{9}. In Caenorhabditis elegans, G6PD deficiency has a strong impact on egg hatching and embryonic development; this is attributed to altered lipid metabolism\textsuperscript{12}. All these data indicate that G6PD is vital in embryonic development and differentiation. However, the underlying mechanism through which G6PD affects embryonic development has not fully been elucidated.

A possible mechanism for G6PD to modulate embryonic development could be mediated by its effect on epithelial–mesenchymal transition (EMT). EMT is a process in which cells transit between the epithelial and mesenchymal states and drives many important aspects of embryonic development\textsuperscript{13}. It is characterized by the loss of epithelial surface markers, which occurs in response to EMT transcription factors or epigenetic regulators. Transforming growth factor β (TGF-β) is vital because of its abilities in the molecular regulation of EMT for the development of metazoans\textsuperscript{14,15}. It is temporally expressed in the developing heart of mice\textsuperscript{16}, and its subfamilies are the key regulators for the generation of axes during embryogenesis\textsuperscript{17}. Data from the Gene Expression Omnibus database indicate that the gene expression of G6PD is downregulated in TGF-β-treated cells (accession nos. GDS3710, GDS2975, and GDS4106). This implies that G6PD may be a factor in regulating the EMT process. The relationship between the G6PD status and EMT process thus warrants investigation.

In the current study, G6PD was identified for the first time as a regulator of the EMT process during embryonic development, demonstrated by cell lines and zebrafish models. G6PD knockdown modulated the expression of EMT markers and zebrafish embryonic development. The modulation of EMT through G6PD knockdown is correlated with the impairment of NOX/Smad3/miR-200b signaling. These data suggest that G6PD plays an important role in regulating embryonic development, as an integral part of the NOX/Smad3/miR-200b axis for modulating the expression of adhesion molecules.

Results

EMT-associated morphological changes in G6PD-knockdown A549 and MDCK cells

To ascertain whether EMT progression is linked to G6PD expression, A549 cells treated with TGF-β were used as cell model. After TGF-β1 treatment for 24 h, a clear morphological change from epithelial (round shape) to mesenchymal (spindle shape) cell types (Fig. 1a) was observed in A549 cells, with E- to N-cadherin switching by decreasing E-cadherin and increasing N-cadherin expression (Fig. 1c). Meanwhile, a decrease in the transcriptional and translational levels of G6PD was detected through polymerase chain reaction (PCR), Western blot, and G6PD activity assays (Fig. 1b, c, and d). To assess whether the morphological differences were associated with cellular G6PD expression, we knocked down G6PD by using lentiviruses to deliver shRNA into two epithelial cell lines, namely A549 and MDCK cells. G6PD-knockdown efficiency was confirmed through protein expression and activity assay (Figs. 1e–f). Compared with the control A549-LG (Lentiviral Scramble control) cells, A549-LG (Lentiviral G6PD shRNA) cells expressed approximately 16% G6PD levels (Fig. 1e). MDCK-LG cells expressed 42% G6PD levels compared with the control MDCK-LS cells (Fig. 1f). G6PD-knockdown cells presented significant alteration in cellular morphology, and both A549-LG and MDCK-LG cells exhibited more mesenchymal-like morphology than did their control cells (Fig. 1g).

Decreased expressions of E-cadherin and β-catenin through G6PD knockdown in A549 and MDCK cells

To further investigate the correlation between G6PD and EMT, the EMT-associated proteins were analyzed in G6PD-knockdown A549 and MDCK cells. The down-regulation of E-cadherin and 8–10% upregulation of N-cadherin were observed in A549-LG and MDCK-LG cells (Fig. 2a). The expression of β-catenin, a subunit of the cadherin protein complex, was also decreased in A549-LG and MDCK-LG cells (Fig. 2a). Moreover, compared with the respective control cells, a reduction in E-cadherin and β-catenin expression and increase in N-cadherin expression at the cell surface were also observed in A549-LG and MDCK-LG cells examined through immunofluorescent staining (Fig. 2b). Using siRNA to further confirm our observation, the gene expression of CDH1 and CTNNB1 were downregulated upon G6PD knockdown concomitant with CDH2 and SNAI1 upregulation in A549 cells (Supplementary Figure 1). These results demonstrate that the inhibition of G6PD expression can reduce cell–cell adhesion through the dysregulation of the adhesion protein complex, which leads to the modulation of EMT.

Enhanced cell migration in G6PD-knockdown A549 cells

Because the downregulation of E-cadherin and β-catenin is often associated with increased cell migration, a scratch wound assay was performed for 24 h. G6PD knockdown was accompanied by enhanced cell migration.
Fig. 1 G6PD knockdown induced morphological changes in epithelial cells. a TGF-β treatment induced morphological changes in A549 cells, accompanied by G6PD inhibition. A549 cells were serum starved for 24 h and treated with 5 ng/mL of TGF-β for 24 h. Compared with the control cells, A549 cells exhibited constricted and mesenchymal-like morphologies after TGF-β stimulation (5 ng/mL). Scale bar, 30 µm. One representative example is shown out of three experiments. b The transcript level of G6PD in TGF-β-treated A549 cells was analyzed through real-time PCR. The relative result was normalized to ACTB, calculated relative to that without the TGF-β treatment counterpart (set to 1 for one experiment), and presented as mean ± SD (**p < 0.01) by performing independently in triplicate. c Protein expressions of G6PD, E-cadherin, and N-cadherin in A549 cells with or without TGF-β stimulation were analyzed through Western blotting. β-Actin was used as the loading control. One representative example is shown out of three experiments. d G6PD activities were determined in A549 cells with or without TGF-β stimulation. Results are expressed as mean ± SD (**p < 0.01). e G6PD expressions and activities of G6PD-knockdown (LG) A549 and control (LS) cells were determined through Western blot and G6PD activity assay. Results are expressed as mean ± SD (**p < 0.01). One representative example is shown out of three Western blot experiments. f G6PD expressions and activities of G6PD-knockdown (LG) MDCK and control (LS) cells were determined through Western blot and G6PD activity assay. Results are expressed as mean ± SD (*p < 0.05). One representative example is shown out of three Western blot experiments. g A549-LG and MDCK-LG cells exhibited constricted and mesenchymal-like morphologies compared with LS cells. Scale bar, 50 µm. One representative example is shown out of four experiments.
 impaired embryonic development of zebrafish by g6pd knockdow

Because EMT plays an important role during embryonic development, the effects of g6pd knockdow on early embryonic development were investigated in a zebrafish model. The optimal delivering dose of g6pd morpholino (MO) were determined following Lund’s group. The kinetics of G6pd activity at 24, 48, and 72 h post-fertilization (hpf) are shown in Supplementary Figure 2. The kinetic data of control MO revealed that G6pd activity was increased during zebrafish embryonic development (Supplementary Figure 2a). However, g6pd MO injection reduced G6pd activity (Supplementary Figure 2a). Compared with the control embryos at 72 hpf, the embryos injected with g6pd MO exhibited a decrease in G6pd activity (41% of control MO; p < 0.05; Fig. 4a) and the inhibition of E-cadherin and β-catenin expression (Fig. 4b). Moreover, the injection of human G6PD cRNA restored the G6pd activity and the expression of E-cadherin and β-catenin in the g6pd morphants (Figs. 4a, b). The expressions of EMT markers were also altered by the G6pd status in zebrafish embryos even at 6 and 16 hpf (Supplementary Figure 3). Decreased expression of β-catenin in g6pd MO embryos could be rescued through the coinjection of human G6PD cRNA but not CDH1 cRNA (Fig. 4b).

E-cadherin is involved in the regulation of zebrafish cell movement and tissue formation during early embryogenesis where loss of E-cadherin decreases epiboly rate and reduces cell–cell adhesion. Additional phenotype experiments were performed to determine whether other EMT-related developmental defects could be observed in the g6pd-knockdown embryos. Indeed, the epiboly rate was decreased (Fig. 4c, up panel), and the change of cell shape at the embryo surface was also observed (Fig. 4c, bottom panel) in g6pd MO-injected embryos compared to control embryos at 6 and 16 hpf, respectively. The coinjection of g6pd MO with human G6PD or CDH1 cRNA rescued these developmental defects (Fig. 4c). All these data indicate that g6pd knockdown influenced the embryonic development of zebrafish through the dysregulation of E-cadherin and β-catenin expression, which led to defective EMT.

Downregulation of E-cadherin and β-catenin through G6PD knockdow via the inhibition of the Smad3/miR-200b pathway

Upon delineating the possible mechanism through which G6PD affects E-cadherin expression, Zeb1, a well-known transcription inhibitor of E-cadherin, was examined. The result showed Zeb1 was upregulated in A549-LG cells compared with in A549-LS cells (Figs. 3a, b, p < 0.05; raw data in Supplementary Table 1). Furthermore, to exclude the effect of proliferation caused by G6PD knockdow, we performed a transwell migration assay. Inhibiting G6PD expression increased migration over a membrane by 59 ± 3.7%, compared with the A549-LG group (Figs. 3c, d, p < 0.05). These enhanced migration data provide further support to the notion that G6PD knockdow can affect EMT.

Fig. 2 The expression of EMT adhesion molecules were altered by the G6PD status in A549 and MDCK cells. a Compared with control (LS) cells, a significant decrease in the protein expressions of E-cadherin and β-catenin and an increase in N-cadherin expression in A549-LG and MDCK-LG cells were observed through Western blot analysis. β-Actin was used as the loading control. One representative example is shown out of three experiments. b Immunofluorescence staining for E-cadherin, N-cadherin, and β-catenin (left, right, and central panels, respectively; green) was observed in A549-LG and MDCK-LG cells compared with in their control LS cells. Nuclei were stained with Hoechest 33342 (blue). Scale bar, 20 µm. One representative example is shown out of three experiments.

by 31 ± 3.6% in A549-LG cells compared with A549-LS cells (Figs. 3a, b, p < 0.05; raw data in Supplementary Table 1).
The reconstitution of SMAD3 in A549-LG cells increased endogenous miR-200b expression (Fig. 5e), downregulated Zeb1 expression, and upregulated the expressions of E-cadherin and β-catenin (Fig. 5f). These results indicate that G6PD knockdown functionally modulates EMT by inhibiting the Smad3/miR-200b pathway.

Upstream mechanism for EMT modulation through G6PD knockdown due to NOX inhibition

Because NOX activation is the key regulator of the Smad3 pathway, the mechanism of the G6PD status in the Smad3 pathway was delineated by analyzing the expressions of NOXs in these cells. The mRNA expression levels of NOX1, NOX2, p22phox, and p67phox, the major NOX subunits in A549 cells, were downregulated in A549-LG cells (data not shown). Because p22phox is the common subunit of NOX1 and NOX2, we further analyzed the protein expression of p22phox in A549-LS and LG cells. As shown in Fig. 6a, the translational level of p22phox was downregulated in A549-LG cells compared with in A549-LS cells. Further study was performed by knocking down p22phox in A549 cells, and decreased protein expressions of Smad3, E-cadherin, and β-catenin, along with upregulated Zeb1, were observed (Fig. 6b). The SNAI1 gene expression was upregulated by p22phox knockdown (Supplementary Figure 4). The level of miR-200b was also downregulated in p22phox-knockdown A549 cells (Fig. 6c), and increased migration over a membrane by 23 ± 5.7%, compared with the scramble control group (Fig. 6d, p < 0.05). These data indicate that the modulation of the EMT process through G6PD knockdown may partly occur due to the downregulation of the NOX/Smad3/miR-200b pathway.

Increased E-cadherin/β-catenin expression by G6PD reconstitution in A549-LG cells through NOX/Smad3/miR-200b signaling

We further reconstituted G6PD expression in A549-LG cells, using Δ1339 as the dominant negative control
A, classic I G6PD deficiency). The transfection of HTG6PD increased the G6PD activity in A549-LG cells, and the transfection of Δ1339 in A549-LG cells did not affect the G6PD activity (Fig. 7a). MiR-200b expression was highly correlated with G6PD activity (Fig. 7b), and G6PD reconstitution upregulated E-cadherin and β-catenin expression and increased the expression of Smad3 and p22phox (Fig. 7c). Moreover, E-cadherin expression could be downregulated by miR-200b LNA and upregulated by ZEB1 siRNA even in the presence of Δ1339 or HTG6PD A549-LG cells (Supplementary Figure 5). Together, these data provide novel information concerning the role of G6PD in modulating the EMT process through the NOX/Smad3/miR-200b pathway.

**Discussion**

G6PD is a well-known housekeeping enzyme whose major function is to maintain cellular redox homeostasis, and studies in knockout mouse reveals that G6PD plays a fundamental role in embryonic development. However, the detailed mechanism regarding why its knockout is embryonically lethal has not been clearly elucidated. The evidence obtained in the current study indicates that G6PD plays an important role in embryonic development, partly by affecting the expression of adhesion molecules. G6pd knockdown in zebrafish can cause EMT or adhesion defects during embryonic development, whereas CDH1 cRNA coinjection can rescue such phenotype. Moreover, G6PD knockdown modulates the EMT.
process by impairing NOX/Smad3/miR-200b signaling. These findings suggest a possible mechanism through which G6PD status affects embryonic development (Fig. 1).

The proposed mechanism concerning G6PD and the embryonic development of zebrafish (Fig. 1) is largely based on the novel findings of the current study, particularly that G6PD knockdown modulates the EMT process. Severe G6PD deficiency leading to embryonic lethality has been well documented. A mouse model has been used to demonstrate that G6PD is indispensable for the development of the embryo and placenta. G6PD(−)

Fig. 5 Inhibition of G6PD downregulated the expression of miR-200b via the Smad3 pathway in A549 cells. a The transcript level of ZEB1 was determined through real-time PCR, normalized to ACTB, and calculated relative to the LS group (set to 1). Results are presented as mean ± SD from three independent experiments (*p < 0.05). b The transcript levels of miR-200b in A549-LS and -LG cells were validated through real-time PCR and normalized to RNU6. The expression level was calculated relative to LS (set to 1) and presented as mean ± SD from three independent experiments (**p < 0.01). c The transcript level of SMAD3 was determined through real-time PCR, normalized to ACTB, and calculated relative to the LS group (set to 1). Results are presented as mean ± SD from three independent experiments (**p < 0.01). d Protein lysates collected from A549-LG and -LS cells were used to analyze the expressions of Zeb1 and Smad3. β-Actin was used as the loading control. One representative example is shown out of three experiments. e Ectopic Smad3 was expressed in SMAD3-overexpressing A549-LG cells, and the transcript levels of miR-200b were analyzed through real-time PCR, normalized to RNU6, and calculated relative to the vector control (set to 1). Data are presented as mean ± SD from three independent experiments (**p < 0.01). f The protein expressions of Smad3, E-cadherin, β-catenin, and Zeb1 were analyzed in SMAD3-overexpressing A549-LG cells. β-Actin was used as the loading control. One representative example is shown out of three experiments.
hemizygous or homozygous embryos stop growing at E8.5 or E7.5, an important stage for establishing blood circulation in mice. G6PD is required for placental development to support the trophoblast, and it plays an embryoprotective role against the development of oxidative stress. The zebrafish model demonstrates that severe g6pd knockdown results in the development of cardiac and kidney edema. Notably, EMT plays a crucial role in embryogenesis. Notably, E-cadherin, a key epithelial surface marker, is downregulated during EMT. The loss of E-cadherin leads to the breaking of cell junctions and contributes to the dissemination of neural crest cells during normal development. E-cadherin expression is also involved in melanocyte development, especially in the migration from the neural crest. β-Catenin is another essential protein in the EMT process, and it regulates cell movements, especially in gastrulation. In the current study, G6PD knockdown impaired E-cadherin and β-catenin expression in cells and in the zebrafish model. Cardiac edema was observed in the g6pd morphants (as indicated by the arrowhead in Supplementary Figure 2b). CDH1 and G6PD cRNA coinjection rescued the developmental defects of g6pd morphants in zebrafish. Taken together, these data provide strong support to the notion that G6PD knockdown impairs embryonic development by influencing the expression of adhesion molecules.

A key player in the proposed pathway involving G6PD and embryonic development is NOX signaling, which uses NADPH as a substrate to produce reactive oxygen species (ROS). NOX family members are responsible for redox signaling, and they have been discovered in the differentiation of various cell types such as neural crest cells, cardiac muscle cells, osteoblasts, macrophages, and renal profibrotic cells. NOX-mediated redox signaling is also involved in cell-to-extracellular matrix interactions such as the anoikis molecular pathways. In the current study, NOX redox signaling modulated by G6PD was involved in the regulation of cell–cell interaction. Although NOX1 and NOX2 are the major isoforms in lung epithelial cells (as reported in another and in our

![Diagram](image.png)

**Fig. 6** Decreased expression of Smad3 in A549-LG cells was due to impaired NOX function. 

- **a** The protein level of p22phox was determined through Western blot analysis in A549-LS and LG cells. β-Actin was used as the loading control. One representative example is shown out of three experiments. 
- **b** A549 cells were transduced with recombinant lentivirus for knocking down p22phox. Ninety-six hours later, the cell lysate was harvested for analysis. The protein expressions of p22phox, Smad3, Zeb1, E-cadherin, and β-catenin were measured through Western blot. β-Actin was used as the loading control. One representative example is shown out of three experiments. 
- **c** The level of miR-200b expression was determined in p22phox-knockdown and control A549 cells through real-time PCR. Data are presented as mean ± SD (n = 3). *p < 0.05. 
- **d** In vitro migration assay was performed to analyze the motility of p22phox-knockdown and control A549 cells. Quantification was conducted by counting migrated cells in five different fields of each independent experiment, and the results are presented as mean ± SD (*p < 0.05).
previous study), NOX4 has also been implicated to be an essential mediator of Smad2/3 transcription factor activation in lung differentiation. G6PD knockdown impaired p22phox expression, the common subunit of NOX1, NOX2, NOX3, and NOX4, and thus decreased the expression of E-cadherin and β-catenin via redox-mediated signaling, possibly by modulating the Smad3/miR-200b pathway. Our data support the notion that G6PD status regulates embryonic development via redox signaling mediated through the NOX pathway. Additional studies are needed to clarify the specific roles of the various isoforms of NOX in redox signaling in different tissues during embryonic development.

The downstream targets in G6PD-mediated NOX signaling are Smad3 and miR-200b. G6PD knockdown decreased Smad3 expression concomitant with miR-200b downregulation (Fig. 2). The Smad pathway has a critical role in the expression of genes controlling embryonic stem cell differentiation. Evidence indicates that Smad proteins inhibit EMT, and Smad3 modulates EMT in a TGF-β-independent manner through the regulation of miR-200 clusters. MiR-200b, which is easily induced by oxidative stress, plays a critical role in modulating EMT, embryonic stem cell differentiation, and the progression of cardiac function. The expression of miR-200b induces the pluripotency of somatic cells through Oct4 and Sox2 upregulation, the two major focuses of stem cell biology. Taken together, G6PD plays a cytoprotective role in producing a low concentration of ROS to affect the Smad3/miR-200b pathway during embryonic development. These data also support the concept that appropriate redox homeostasis is essential for controlling embryonic development.

Another novel observation in the current study highlights that G6PD knockdown downregulated β-catenin expression. β-Catenin has a dual function in gene transcription and cell–cell adhesion. Alignment analysis was used to compare the protein sequences in humans (Homo sapiens) and zebrafish (Danio rerio), and >90% identical sequences over entire β-catenin lengths were discovered (Supplementary Table 2). The homological identities of G6PD and E-cadherin were 76.4 and 48.1% between these two species, respectively. β-Catenin is the
key regulator for directing several developmental processes, especially via Wnt signaling, and the formation of different body regions, such as those involving cardiac physiology\textsuperscript{52,53}. Furthermore, $\beta$-catenin has also been implicated in regulating the differentiation of mesodermal cell lineages such as macrophages and the asymmetric cell division of \textit{C. elegans}. We noted that \textit{G6PD} knockdown inhibited $\beta$-catenin expression, and cardiac edema occurred in the \textit{g6pd} morphants of zebrafish. Moreover, \textit{G6PD} knockdown can lead to the loss of asymmetric cell division in the embryos of \textit{C. elegans}\textsuperscript{12} and can prohibit macrophage differentiation (our unpublished data). These data indicate that the \textit{G6PD} status affects cell differentiation and embryonic development, partly via the $\beta$-catenin pathway.

Our novel findings indicate that \textit{G6PD} plays a regulatory role in modulating EMT, which could have major clinical implications in carcinogenesis. Cancer cells exhibit increased glycolysis and pentose cycle activity compared with normal cells. These metabolic alternations were thought to arise from the damage to the respiratory mechanism, and cancer cells were thought to compensate for this defect by increasing glycolysis. An increase in \textit{G6PD} activity is observed in most cancer cell lines, and this promotes cell proliferation. Accordingly, mutations leading to the overexpression of $\beta$-catenin are closely associated with cancer progression\textsuperscript{54}. This may explain why \textit{G6PD} upregulation is correlated with cancer progression through the upregulation of $\beta$-catenin signaling. By contrast, Anelisa et al. used proteomic analysis to demonstrate that tumorigenic glioblastomas have low \textit{G6PD} protein expression and increased cell migration\textsuperscript{55}. Our observations that \textit{G6PD} knockdown impairs $\beta$-catenin expression but enhances cell migration are consistent with those of Anelisa et al.\textsuperscript{55}. Because cancer progression is complicated with the participation of multiple factors, the role of \textit{G6PD} in carcinogenesis needs to be further delineated.

In summary, our study demonstrated that \textit{G6PD} is a common feature for regulating cell-cell interactions by modulating the expression of adhesion molecules. The current study also indicates that \textit{G6PD} plays a cytorregulatory role in redox signaling by influencing the NOX pathway, which is critical to many cellular functions. The impaired NOX/Smad3/miR-200b axis is, in part, the mechanism through which \textit{G6PD} knockdown impairs E-cadherin and $\beta$-catenin expression. Taken together, these data strongly suggest that in embryonic development, \textit{G6PD} plays an essential role as an integral component of the NOX/Smad3/miR-200b axis.

\textbf{Materials and methods}

\textbf{Materials}

The following antibodies were used: anti-G6PD (Genesis Biotech Inc., New Taipei, Taiwan), anti-E-cadherin, anti-N-cadherin, anti-$\beta$-catenin (BD Biosciences, San Jose,
CA, USA), anti-β-actin (GeneTex Inc., Irvine, CA, USA), alexa fluor 488 goat anti-mouse IgG, alexa fluor 594 phalloidin (Thermo Fisher Scientific, Waltham, MA, USA), anti-ZEB1, anti-Smad3 (Cell Signaling Technology, Danvers, MA, USA), anti-p22phox (Abcam, Cambridge, UK), horseradish peroxidase (HRP)-conjugated anti-mouse IgG, and anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Recombinant human TGF-β1 was purchased from PeproTech (Rocky Hill, NJ, USA). The 69pd targeting and nontarget Morphinolinos were purchased from Gene Tools (Philomath, OR, USA).

**Cell culture**

MDCK cells were kindly provided by Dr. Shih Sin-Ru at the Chang Gung University and were cultured in Dulbecco’s modified Eagle’s media containing 10% fetal bovine serum (FBS). Human adenocarcinoma epithelial cell lines (A549) were purchased from ATCC and cultured in DMEM supplemented with 10% FBS. These cells were maintained in a humidified atmosphere of 5% CO2 at 37 °C. Thawed cells from liquid nitrogen were used for within the first five passages.

**Plasmid construction and transduction by lentivirus system**

pLKO.1.null-T and pLAS2w.Ppuro plasmids were purchased from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). The mini-cassette encoding shRNA against G6PD, p22phox, or scramble gene include overhangs that are allowed cloning into pLKO.1.null-T. Scramble shRNA was used as a negative control. The sequence of G6PD shRNA cassettes were (sense) 5′-CCGGGATACACACATATTCATCATCCTCGAGGA TGATGAAATGTGGTGATCCTTTTT-3′ and (anti-sense) 5′-AAATGGGAAGGATACACACATATTCATCATCCTCGAGGA TGATGAAATGTGGTGATCCTTTTT-3′. The sequence of p22phox shRNA cassettes were (sense) 5′-CCGGGATACATGACCGCCGTGGTAAGCTCAGCTCGAGTT CACCCAGCAGGCTCATGTATTTT-3′ and (anti-sense) 5′-AAATGGGAAGGATACACACATATTCATCATCCTCGAGGA TGATGAAATGTGGTGATCCTTTTT-3′. The sequence of scramble shRNA cassettes were (sense) 5′-CCGGGATACATGACCGCCGTGGTAAGCTCAGCTCGAGTT CACCCAGCAGGCTCATGTATTTT-3′ and (anti-sense) 5′-AAATGGGAAGGATACACACATATTCATCATCCTCGAGGA TGATGAAATGTGGTGATCCTTTTT-3′. The sequence of scramble shRNA cassettes were (sense) 5′-CCGGGATACATGACCGCCGTGGTAAGCTCAGCTCGAGTT CACCCAGCAGGCTCATGTATTTT-3′ and (anti-sense) 5′-AAATGGGAAGGATACACACATATTCATCATCCTCGAGGA TGATGAAATGTGGTGATCCTTTTT-3′. The sequence of scramble shRNA cassettes were (sense) 5′-CCGGGATACATGACCGCCGTGGTAAGCTCAGCTCGAGTT CACCCAGCAGGCTCATGTATTTT-3′ and (anti-sense) 5′-AAATGGGAAGGATACACACATATTCATCATCCTCGAGGA TGATGAAATGTGGTGATCCTTTTT-3′. The plasmid (name as HTG6PD or SMAD3). Δ1339 was constructed by KOD plus polymerase (TOYOBO CO., Osaka, JP) using forward and reverse mutagenesis primers: (Forward) 5′-TGGACGCTTCTTGTGCGAGACCAGTTCTTCAACACTGTTAACAATACATATCTCAGGATAATGTTGCAAGTGTAGTTGAGTCGAGCTTGACCCAACCGGTACTATTTT-3′ and (anti-sense) 5′-GGACGCTTCTTGTGCGAGACCAGTTCTTCAACACTGTTAACAATACATATCTCAGGATAATGTTGCAAGTGTAGTTGAGTCGAGCTTGACCCAACCGGTACTATTTT-3′. The plasmid encoding human His-tagged G6PD was designed according to the sequences of human gene cDNAs and miRNA genes. The sequences of the primers used in reverse transcription and real-time PCR analysis were designed to yield the extract. Samples were denatured, electrophoresed on 10% SDS-polyacrylamide gel, and transferred to PVDF membranes. The membranes were incubated overnight at 4 °C with an appropriate dilution of a primary antibody (1:1000) in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, 0.05% SDS, 150 mM NaCl, 1 mM EGTA and 1 mM NaF). Cell lysate was centrifuged and the supernatant was used in the assay. G6PD activity was analyzed by combining protein and assay buffer (50 mM Tris–HCl (pH 8), 50 mM MgCl2, 4 mM NADP+, and 4 mM glucose 6-phosphate).

**Real-time PCR analysis**

Total RNA of cells was isolated using Trizol reagent and reverse transcription was performed using the SuperScript III system (Invitrogen, Carlsbad, CA, USA). Primers were designed according to the sequences of human gene cDNAs and miRNA genes. The sequences of the primers used in reverse transcription and real-time PCR are listed in Supplementary Table 3. Real-time PCR was performed using the IQ™ SYBR Green Supermix kit on an IQ5 Real Time Thermal Cycler (Bio-Rad, Hercules, CA, USA). Relative fold expression values were determined using the
Δ△Ct method. The gene expression levels were normalized to the level of the reference gene (ACTB) and miRNA expression level was normalized to the level of small nuclear RNA U6. All experiments were performed independently at least in triplicate.

Immunofluorescence
A549 and MDCK cells were cultured in glass slides. After confluency, cells were fixed with 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. Cells were incubated with mouse monoclonal anti-E-cadherin (1:50, BD Biosciences, San Jose, CA, USA), mouse monoclonal anti-β-catenin (1:200, BD Biosciences, San Jose, CA, USA), mouse monoclonal anti-N-cadherin (1:50, BD Biosciences, San Jose, CA, USA) overnight at 4 °C, and they were washed three times with PBS for 15 min each. Subsequently, the cells were incubated with Alexa fluor 488 goat anti-mouse IgG secondary antibodies (1:100 for E- and N-cadherin and 1:400 for β-catenin, Thermo Fisher Scientific, Waltham, MA, USA) and with 2 μg/ml of Hoechst 33342 dye for nuclear staining for 60 min at room temperature. After incubation, the samples were rinsed 3 times with PBS for 15 min each. Finally, the samples were examined under an inverted fluorescence microscope (Olympus, Tokyo, JP).

Migration assays and wound-healing experiment
In vitro migration assays, a total of 8 × 10^4 cells in 200 μl serum-free Dulbecco’s modified Eagle (DMEM) media were plated onto Millicell culture plate inserts (8 μm pore size; Merk Millipore, Darmstadt, Germany), and the lower chamber was immediately filled with 500 μl of DMEM media with 10% FBS as a chemoattractant. After 24 h of incubation in a humidified atmosphere containing 5% CO2 at 37°C, the membranes were then fixed with methanol and stained by 0.2% crystal violet. Each individual experiment had triplicate inserts, and 5 microscopic fields were counted per insert. For wound-healing experiments, cells were plated in 6-well plates and cultured for confluency. Cells were serum-starved and scraped with a P200 pipette at (time 0), and pictures were taken at the 24 h time points then analyzed objectively using Image J. The percentage of wound closure at 24 h were measured by the size of the wound relative to that at 0 h in triplicate and the data were presented as mean ± SD.

Ethics statement and fish maintenance
All experiments were performed in accordance with standard guidelines for zebrafish work and approved by the Institutional Animal Care and Use Committee of Chang Gung University (IACUC approval number: CGU16-045). Tü (wild type) zebrafish embryos were purchased from the Zebrafish International Resource Center (Oregon) and were raised, maintained, and paired under standard conditions. The embryos were staged according to the number of somites, hours postfertilization, and days postspermatization.

RNA and morpholino injection
Capped RNA encoding the full coding sequences of human E-cadherin and G6PD were prepared as described previously. Antisense morpholino (MO) oligonucleotides were purchased from Gene Tools (Philomath, OR, USA). The sequences of g6pd translation blocker and splice MOs were designed as the materials in previous study. In brief, the g6pd MOs were combined in a 1:1 ratio for delivery of 0.13 pmol for knockdown injection. Random MOs at 0.13 pmol were used for control injection. All injections were performed at the one- to two-cell stage and cRNAs or morpholinos were introduced into blastomeres.

Statistical analysis
All data were expressed as means ± SD from replicated determinations. The student t test was used to compare the mean values from the two groups. Difference were regarded as significant if the p value was <0.05.

Acknowledgements
This work is made possible by grants from the Ministry of Science and Technology of Taiwan (MOST105-2320-B-182-031-MY2 to DTYC), from the Ministry of Education of Taiwan (EMRPD1G0181 to CGU), and from Chang Gung Memorial Hospital (BMRP098 and CMRPD1F0461 to DTYC). We also thank miRNA plate form at Chang Gung University. This manuscript was edited by Wallace Academic Editing.

Author details
1Research Center for Chinese Herbal Medicine, College of Human Ecology, Chang Gung University of Science and Technology, Taoyuan, Taiwan.
2Department of Medical Biotechnology and Laboratory Science, College of Biomedical Sciences, College of Medicine, Chang Gung University, Taoyuan, Taiwan.
3Graduate Institute of Biomedical Sciences, College of Medicine, Chang Gung University, Taoyuan, Taiwan.
4Department of Pediatric Hematology/Oncology, Linkou Chang Gung Memorial Hospital, Taoyuan, Taiwan.
5Neuroscience Research Center, Chang Gung Memorial Hospital at Linkou Medical Center, Taoyuan, Taiwan.
6Healthy Aging Research Center, Chang Gung University, Taoyuan, Taiwan.

Authors’ contributions
YHW, YCC, and DTYC co-designed the experiments with help from SHC; experiments were performed by YHW, YHL, and HYS. All authors analyzed the results, wrote the manuscript, contributed substantially to the present work, then read and approved the final manuscript.

Competing interests
The authors declare that they have no competing financial interests.

Publisher’s note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary information
The online version of this article contains supplementary material.

Official journal of the Cell Death Differentiation Association
References

1. Cappellini, M. D. & Fiorelli, G. Glucose-6-phosphate dehydrogenase deficiency. Lancet. 371, 64–74 (2003).
2. Luzzatto, L. & Seneca, E. G6PD deficiency: a classic example of pharmacogenetics with on-going clinical implications. Br. J. Haematol. 164, 489–490 (2014).
3. Wan, G. H., Tsai, S. C. & Chiu, D. T. Decreased blood activity of glucose-6-phosphate dehydrogenase associates with increased risk for diabetes mellitus. Endocrine. 19, 191–195 (2002).
4. Gaskin, R. S., Estwick, D. & Peddi, R. G6PD deficiency: its role in the high prevalence of hypertension and diabetes mellitus. Ehrn. Dis. 11, 749–754 (2001).
5. Lin, H. R. et al. Proteome-wide dysregulation by glucose-6-phosphate dehydrogenase (G6PD) reveals a novel protective role for G6PD in aflatoxin B1-mediated cytotoxicity. J. Proteome Res. 12, 3434–3448 (2013).
6. Lin, H. R., Wu, Y. H., Yen, W. C., Yang, C. M. & Chiu, D. T. Diminished COX-2/PGE2-Mediated Antiviral Response Due to Impaired NO/MAPK Signaling in G6PD-Knockdown Lung Epithelial Cells. PLoS. ONE. 11, e0153962 (2016).
7. Wu, Y. H. et al. Glucose-6-Phosphate Dehydrogenase Enhances Antiviral Response through Downregulation of NADPH Sensor HSCARG and Upregulation of NF-κB Signaling. Viruses. 7, 6698–6706 (2015).
8. Tang, H. Y. et al. Inability to maintain GSH pool in G6PD deficiency: a classic example of pharmacogenetics with on-going clinical implications. Br. J. Haematol. 164, 489–490 (2014).
9. Longo, L. et al. Maternally transmitted severe glucose 6-phosphate dehydrogenase deficiency is an embryonic lethal. EMBO J. 21, 4229–4239 (2002).
10. Yang, H. C., Wu, Y. H., Liu, H. Y., Stern, A. & Chiu, D. T. Whathapass e disproloq: 2016.
11. Nieto, M. A., Huang, R. Y., Jackson, R. A. & Thiery, J. P. Emt:2016.
12. Yang, J. & Weinberg, R. A. Epithelial-mesenchymal transition: at the crossroads of cell motility and contact repulsion in Drosophila macrophages in vivo. J. Cell Biol. 189, 681–689 (2010).
13. Nishimura, E. K., Yoshida, H., Kunisada, T. & Nishikawa, S. I. Regulation of E- and P-cadherin expression correlated with melanocyte migration and diversification. Dev. Biol. 215, 155–166 (1999).
14. Mort, R. L., Jackson, J. J. & Patton, E. E. The melanocyte lineage in development and disease. Development. 142, 1387 (2015).
15. Shimizu, T. et al. E-cadherin is required for gastrulation cell movements in zebras. Mech. Dev. 122, 747–765 (2005).
16. Larabell, C. A. et al. Establishment of the dorsal-ventral axis in Xenopus embryos is persaged by early asymmetries in beta-catenin that are modulated by the Wnt signaling pathway. J. Cell Biol. 136, 1123–1136 (1997).
17. Lee, J. E., Cho, K. E., Lee, K. E., Kim, J. & Bae, Y. S. Nox4-mediated cell signaling regulates differentiation and survival of neural crest stem cells. Mol. Cells. 37, 907–911 (2014).
18. Nadworny, A. S. et al. Nox2 and Nox4 influence neonatal k+(-) cardiac precursor cell state and differentiation. Am. J. Physiol. Cell. Physiol. 305, H829–H842 (2013).
19. Mandal, C. C. et al. Reactive oxygen species derived from Nox4 mediate BMP2 gene transcription and osteoblast differentiation. Biochem. J. 433, 393–402 (2011).
20. Xu, Q. et al. NADPH oxidases are essential for macrophage differentiation. J. Biol. Chem. 291, 20039–20041 (2016).
21. Simone, S. et al. BMP-2 induces a profibrotic phenotype in adult renal progenitor cells through Nox4 activation. Am. J. Physiol. Renal. Physiol. 303, F23–F34 (2012).
22. Chiarugi, P. & Giannoni, E. Anoikis: a necessary death program for anchorage-dependent cells. Biochem. Pharmacol. 76, 1532–1536 (2008).
23. Groeger, G., Quiryn, C. & Cotter, T. G. Hydrogen peroxide as a cell-survival signaling molecule. Antioxid. Redox Signal. 11, 2765–2767 (2009).
24. Paoli, P., Giannoni, E. & Chiarugi, P. Anoikis molecular pathways and its role in cancer progression. Biochem. Biophys. Acta. 1833, 3481–3498 (2013).
25. Fink, K., Duval, A., Martel, A., Soucy-Faulkner, A. & Grandvaux, N. Dual role of NOx2 in respiratory syncytial-virus- and Sendai virus-induced activation of NF-κB and NF-κB in airway epithelial cells. J. Immunol. 180, 6991–6992 (2008).
26. Amara, N. et al. NOx4/NADPH oxidase expression is increased in pulmonary fibroblasts from patients with idiopathic pulmonary fibrosis and mediates TGFbeta1-induced fibroblast differentiation into myofibroblasts. Thorax. 65, 733–738 (2010).
27. Ahn, S. M. et al. Smad3 regulates E-cadherin via miR-200A pathway. Oncogene. 31, 3051–3059 (2012).
28. Moustakis, A., Souchelnytskyi, S. & Heldin, C. H. Smad regulation in TGF-beta signal transduction. J. Cell. Sci. 114Pt 24, 4359–4369 (2001).
29. Masague, J. & Xi, Q. TGF-beta control of stem cell differentiation genes. FEBS Lett. 586, 1953–1958 (2012).
30. Pohl, M. et al. SMAD4 mediates mesenchymal-epithelial reversion in SW480 colon carcinoma cells. Am. J. Pathol. 160, 2603–2613 (2000).
31. Zheng, J. et al. Retinoic Acid Induces Embryonic Stem Cell Differentiation by Altering Both Encoding RNA and microRNA Expression. PLoS. ONE. 10, e0132566 (2015).
32. Feng, B. et al. miR-200B mediates Endothelial-to-Mesenchymal Transition in Diabetic Cardiomyopathy. Diabetes. 65, 768–779 (2016).
33. Wang, G. et al. Critical regulation of miR-200ZEB2 pathway in Oct4/Sox2-induced mesenchymal-to-epithelial transition and induced pluripotent stem cell generation. Proc. Natl. Acad. Sci. U. S. A. 110, 2858–2863 (2013).
34. Rizzino, A. Concise review: The Sox2-Oct4 connection: critical players in a much larger interdependent network integrated at multiple levels. Stem Cell. 31, 1033–1039 (2013).
35. Brembeck, F. H., Rosario, M. & Birchmeier, W. Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. Curr. Opin. Genet. Dev. 16, 51–59 (2006).
36. Sokol, S. Y. Spatial and temporal aspects of Wnt signaling and planar cell polarity during vertebrate embryonic development. Semin. Cell. Dev. Biol. 42, 76–85 (2015).
53. Grainger, S. et al. Wnt9a Is Required for the Aortic Amplification of Nascent Hematopoietic Stem Cells. Cell Rep 17, 1595–1606 (2016).
54. Morin, P. J. beta-catenin signaling and cancer. Bioessays 21, 1021–1030 (1999).
55. Ramao, A. et al. Changes in the expression of proteins associated with aerobic glycolysis and cell migration are involved in tumorigenic ability of two glioma cell lines. Proteome. Sci. 10, 53 (2012).
56. Halpern, M. E. et al. Cell-autonomous shift from axial to paraxial mesodermal development in zebrafish floating head mutants. Development 121, 4257–4264 (1995).
57. Chung, P. C. et al. Zebrafish Her8a is activated by Su(H)-dependent Notch signaling and is essential for the inhibition of neurogenesis. PLoS ONE 6, e19894 (2011).