Proteomic analysis of Nrk gene-disrupted placental tissue cells explains physiological significance of NRK

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Abstract
Objective: NRK is a unique X chromosome-linked protein kinase expressed predominantly in placenta. The gene knockout causes placental overgrowth and delayed labor of Nrk-null fetuses from dams in mouse. To clarify unknown mechanisms behind the Nrk-null phenotypes, protein expression profiles were analyzed in the Nrk-null placenta using a high-performance two-dimensional electrophoresis methodology.

Results: Among around 1800 spots detected, we characterized a dozen protein spots whose expression levels were significantly altered in the Nrk-null placenta compared to wild-type. Analyzing these data sets is expected to reflect the difference physiologically in the presence or absence of NRK, facilitating the development of therapeutic strategies.

Keywords: Placentomegaly, Dystocia, Breast tumor, Placenta, Protein kinase

Introduction
Parturition essential for the survival and proliferation of eutherian mammals is considered to be strictly regulated for ensuring the safety of the next-generation descendants [1, 2]. During pregnancy, the fetus and placenta are most likely to communicate with each other until delivery in seeking the safe and secure opportunity for birth. However, little is known about the shared mechanism that controls communication between the mother and the unborn. By analyzing various single-gene knockout (KO) mice, several candidates of the messaging molecules involved in the negotiation for selecting the best timing of birth have been identified to date [3–9]. One of the most prominent of these candidates is NRK [10].

NRK (NIK-related kinase), highly expressed in the placenta, is a physiologically unique X-encoded Ser/Thr protein kinase [10–12]. We previously reported that the Nrk gene KO causes placental overgrowth, indicating that NRK is a crucial modulator of cell proliferation and development in placental tissues [10]. Furthermore, the Nrk-null fetuses influence the pregnant dam to delay delivery. Together with subsequent work using intrauterine embryonic transfer of Nrk-null fetuses into wild-type (WT) dams [10], these results suggested that NRK is required for mediating one or more unidentified delivery-inducing signals dispatched from the placenta. In addition, we have found that Nrk mutant female mice develop breast tumors frequently, suggesting that NRK is a tumor-suppressor gene [13].

These results tempted us to clarify the molecular mechanisms behind the Nrk-null phenotypes by using proteomic analysis to profile the protein expression of key regulators in the placenta of Nrk KO fetuses. We presented herein detailed two-dimensional electrophoresis (2DE) reference maps of the mutant mouse placenta to establish a NRK-connected placental database, available worldwide, that contains information on protein species identified by 2DE.
**Main text**

**Experimental methods**

We applied a high-performance 2DE methodology to analyse mouse placenta harvested in the third trimester of pregnancy. Variations in protein expression levels were defined by comparing individual protein spots on the resulting gels. The trophoblast tissue layer samples were subsequently dissected from the whole placenta as in Additional file 1: Figure S1. After treating the samples using a 2-D Clean-Up Kit (GE Healthcare Ltd., UK), protein quantification, isoelectric focusing of proteins, 2DE, gel-staining, and data analysis were performed as described by Wong et al. [14].

**Results and discussion**

In Table 1, we calculated the individual average from six independent trials of 2DE (Fig. 1). Although some variations are observed, it is considered that the increase or decrease in protein amount of each spot has been verified. Among several identified proteins whose levels were attenuated in the mutant, annexin A3 and A5 belong to the annexin family composed of functionally diverged Ca²⁺-dependent membrane phospholipid-bound intracellular proteins [15]. Downregulation of annexin A3 (Spot 5) inhibits growth, migration, invasion, and metastasis of lung cancer cells by suppressing the MEK/ERK signaling pathway [16]. Annexin A3 may be involved in

| Spot Nr | Protein description | Protein entry | Accession | Score | avgMass | seqCover (%) | Effect size | SD | t test (n = 6) |
|---------|---------------------|---------------|-----------|-------|---------|--------------|-------------|----|---------------|
| 1       | Protein 2210010C04Rik OS Mus musculus GN 2210010C04Rik PE 2 SV 1 | Q9CPN9_MOUSE | Q9CPN9 | 435   | 26422   | 8.10         | —           | 0.710 | 0.653 0.009   |
| 2       | Heat shock protein HSP 90 beta OS Mus musculus GN Hspa9b1 PE 1 SV 3 | HS90B_MOUSE | P11499 | 102   | 83281   | 3.59         | —           | 0.248 | 0.126 0.020   |
| 3       | Stress 70 protein mitochondrial OS Mus musculus GN Hspa9 PE 1 SV 3 | GRP75_MOUSE | P38647 | 1030  | 73461   | 39.62        | —           | 0.018 | 0.774 0.942   |
| 4       | Protein Serpinb9f OS Mus musculus GN Serpinb9f PE 2 SV 1 | Q80UK5_MOUSE | Q80UK5 | 535   | 43034   | 16.98        | —           | 0.126 | 0.096 0.148   |
| 5       | Annexin A3 OS Mus musculus GN Anxa3 PE 1 SV 4 | ANXA3_MOUSE | O35639 | 556   | 36384   | 27.24        | —           | 0.127 | 0.169 0.062   |
| 6       | Staphylococcal nuclease domain containing protein 1 OS Mus musculus GN Snd1 PE 1 SV 1 | SND1_MOUSE | Q78P8Y7 | 50    | 102088  | 4.95         | —           | 0.109 | 0.682 0.846   |

Table 1 List of mouse placental proteins whose expression differed significantly between control and Nrk⁻/⁻ 2DE performed on proteins from E18.5 concepti, as identified by LC-MS/MS analysis

Specified proteins of the Nrk⁻/⁻ mouse placenta are indicated

| Spot Nr | Protein description | Protein entry | Accession | Score | avgMass | seqCover (%) | Effect size | SD | t test (n = 6) |
|---------|---------------------|---------------|-----------|-------|---------|--------------|-------------|----|---------------|
| 7       | –                   | –             | –         | –     | –       | —            | —           | 0.285 | 0.555 0.492   |
| 8       | Protein 2210010C04Rik OS Mus musculus GN 2210010C04Rik PE 2 SV 1 | Q9CPN9_MOUSE | Q9CPN9 | 727   | 26422   | 4.86         | —           | 0.309 | 0.233 0.031   |
| 9       | Pyruvate kinase isozymes M1 M2 OS Mus musculus GN Pkm PE 1 SV 4 | KPYM_MOUSE | P52480 | 3716  | 57845   | 50.66        | —           | 0.272 | 0.498 0.294   |
| 10      | –                   | –             | –         | –     | –       | —            | —           | 0.221 | 0.347 0.198   |
| 11      | Protein disulfide isomerase A5 OS Mus musculus GN Pdia5 PE 2 SV 1 | PDIAS5_MOUSE | Q921X9 | 302   | 59267   | 15.67        | —           | 0.666 | 0.816 0.116   |
| 12      | Glutamate dehydrogenase 1 mitochondrial OS Mus musculus GN Glud1 PE 1 SV 1 | DHE3_MOUSE | P26443 | 176   | 61337   | 10.75        | —           | 0.021 | 0.539 0.654   |

Specified proteins of the Nrk⁻/⁻ mouse placenta are indicated

| Spot Nr | Protein description | Protein entry | Accession | Score | avgMass | seqCover (%) | Effect size | SD | t test (n = 6) |
|---------|---------------------|---------------|-----------|-------|---------|--------------|-------------|----|---------------|
| 13      | T complex protein 1 subunit alpha OS Mus musculus GN Tcop1 PE 1 SV 3 | TCPA_MOUSE | P11983 | 465   | 60449   | 30.22        | —           | 0.217 | 0.271 0.208   |
| 14      | 40S ribosomal protein SA OS Mus musculus GN Rpsa PE 1 SV 4 | RPSA_MOUSE | P14206 | 3256  | 32838   | 47.46        | —           | 0.110 | 0.148 0.115   |
| 15      | Annexin A5 OS Mus musculus GN Anxa5 PE 1 SV 1 | ANXA5_MOUSE | P48036 | 12996 | 35753   | 83.39        | —           | 0.114 | 0.179 0.223   |
| 16      | SPARC OS Mus musculus GN Sparc PE 1 SV 1 | SPARC_MOUSE | P07214 | 126   | 34450   | 11.59        | —           | 0.256 | 0.597 0.235   |
| 17      | Calpain small subunit 1 OS Mus musculus GN Capn1 PE 2 SV 1 | CPN1_MOUSE | O88456 | 951   | 28463   | 31.97        | —           | 0.068 | 0.303 0.531   |
| 18      | Annexin A2 OS Mus musculus GN Anxa2 PE 1 SV 2 | ANXA2_MOUSE | P07356 | 14770 | 38676   | 67.26        | —           | 0.102 | 0.410 0.509   |

Specified proteins of the Nrk⁻/⁻ mouse placenta are indicated

| Spot Nr | Protein description | Protein entry | Accession | Score | avgMass | seqCover (%) | Effect size | SD | t test (n = 6) |
|---------|---------------------|---------------|-----------|-------|---------|--------------|-------------|----|---------------|
| 19      | –                   | –             | –         | –     | –       | —            | —           | 0.285 | 0.555 0.492   |

* Individual effect size is an average calculated from six independent trials

* The protein spots corresponding spot number 7 and 10 could not be specified
the metabolism of estrogens; this function could be relevant, given that estrogen-induced disruption of the intracellular microenvironment leads to membrane damage and cell cycle arrest [17]. Annexin A5 (Spot 15) is known to be an expedient diagnostic marker for detecting apoptotic cells that is a component of the outer leaflet of the plasma membrane [18]. Decreased levels of Annexin A5 in the Nrk KO placenta tempts us to speculate that NRK functions by potentiating cell death, thereby promoting excess proliferation of specific tissue layers without NRK activity, and leading to phenotypes such as placentomegaly and breast tumorigenesis.

Calpain, a calcium-regulated cysteine protease corresponded to Spot 17, the intensity of which was decreased in the Nrk KO placenta. Calpain is implicated in cytoskeletal remodeling and signal transduction. Calpain-mediated proteolytic cleavage induces cytoskeletal dynamics. This activity is altered during aging and in the progression of numerous diseases, including calcium-dependent disorders and pathological conditions [19, 20]. Spot 2, a signal decreased in the KO placenta, was identified as HSP90, a molecular chaperone with numerous client proteins. Many HSP90 inhibitors are undergoing study for potential use as clinical therapies [21]. Calpain is known to regulate HSP90 expression by cleaving HSP90 directly. On the other hand, HSP90 has been reported to affect the activity of calpain, suggesting that interactions between HSP90 and calpain jointly contribute to physiological functions. Indeed, genetic disruption of the calpain-encoding gene or treatment with HSP90 inhibitors has been shown to yield attenuation of mammary tumorigenesis [22].

Spot 4, a signal decreased in the KO placenta, corresponded to serpins of clade B (serpinb9f), a unique class of intracellular protease inhibitors [23]. Among these inhibitors, serpin B9 is a well-studied specific inhibitor of granzyme B [24]. It seems likely that the granzyme-mediated proteolysis is important for the immune response to infection or tumorigenesis. Changes in serpin levels are expected to cause cell damage in normal tissues. Therefore, NRK may contribute to a cytoprotective function by safeguarding lymphocytes from granzymes.

For the purposes of the present study, proteomics may be a more informative approach than gene expression (transcriptional) profiling, given that transcript accumulation does not always correlate with qualitative or quantitative differences in protein levels and often fails to reflect in vivo protein localization, depending on the tissue. Our data tempted to speculate that dysfunction of NRK leads to defects in cellular proliferation, cell cycle progression, resistance to apoptosis, and oncogenesis. Also, recent progress in advanced mass spectrometry methods is expected to enable us to monitor numerous phosphorylation sites in proteins. Together with the genomic discoveries through genome-wide association studies reported recently
[25], further profiling analyses of the gene product changes in the Nrk-gene-mutants is expected to clarify the functional mechanism of fetoplacental development and differentiation during pregnancy, facilitating the identification of potential targets of current chemotherapeutic treatments available for perinatal medicine.

Limitations
The main limitation of our research was that we couldn’t verify why each identified expressed protein decreased in NRK-deficient placental tissue cells which could explain how NRK works physiologically in the state of health. Elucidating the physiological role of NRK in future studies would not only become one target protein for drug discovery but also helps to improve human health.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s13104-019-4818-7.

Abbreviations
2DE: two-dimensional gel electrophoresis; dpc: days postcoitum; ER: estrogen receptor; H&E: hematoxylin and eosin; LC-MS/MS: liquid chromatography-tandem mass spectrometry; PCR: polymerase chain reaction; WT: wild-type; KO: knockout.

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Authors’ contributions
KD, AJ, KNW, MK and NH conceived the study; KD, KI, KNW, MK and NH generated and analyzed experimental data; MT, MK and NH provided a critical review of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate
All animal experiments were approved by and performed under the guidelines of the Institutional Animal Care and Research Advisory Committee of Tokyo Institute of Technology.

Consent for publication
Not applicable.

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

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