主論文の要旨

# Fetal growth restriction followed by early catch-up growth impairs pancreatic islet morphology in male rats

（雄のラットでは胎児発育不全に早期成長キャッチアップが続くと膵島の形態を障害する）

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## 【Introduction】

Fetal growth restriction (FGR) is a condition wherein a fetus does not reach its biological growth potential, mainly due to placental insufficiency. FGR affects around 30 million newborns yearly and remains a major cause of perinatal mortality and morbidity. It is associated with risks of adverse health outcomes in later life including type 2 diabetes, and obesity. Several FGR animal models have been studied to explore the impact of FGR on postnatal development including protein restriction caused impaired islet development and functional alterations in pancreatic islets. Epidemiological studies showed that FGR due to placental insufficiency increases the risk of type2 diabetes because of impaired β-cell development during the perinatal period.

We hypothesized that FGR, followed by good postnatal nutrition, affects postnatal growth, blood glucose levels, serum lipid levels, and blood pressure; such effects might influence pathogenesis of type 2 diabetes.

We sought to determine the effects of FGR and postnatal catch-up growth on the development of type2 diabetes. We focused on islet morphology and fibrosis, and determining which mechanisms of pancreatic protein dysregulation were responsible for the FGR-induced morphological changes in the pancreatic islets, followed by a period of early, postnatal catch-up growth. We used our recently developed rat FGR model of chronic ischemia *in utero* since it more closely mimics placental insufficiency.

## 【Results】

### Body weight

Compared to sham-operated dams, the birth litter size of FGR-operated dams was reduced by 52% ($P < 0.001$) and birth weight by 20.9%.

### Intraperitoneal glucose tolerance test (IPGTT)

At 8 weeks, blood glucose levels were higher in male FGR good nutrition (FGR-GN) group rats compared to male sham poor nutrition (sham-PN) group and sham good nutrition (sham-GN) group rats. The blood glucose levels were higher in the FGR poor nutrition (FGR-PN) group than in the sham-GN group ($P < 0.05$) (Figure 2c). At 24 weeks, blood glucose levels were higher in the FGR-GN group than in the sham-GN, sham-PN ($P < 0.01$), or FGR-PN groups ($P < 0.05$) (Figure 2d).

On the other hand, among 8-week-old female rats, blood glucose levels were higher in the FGR-PN than in the sham-GN ($P < 0.01$) or the sham-PN groups ($P < 0.05$). The FGR-GN group had a higher average blood glucose level than the sham-GN group ($P < 0.05$) (Figure 2e). At 24 weeks, the blood glucose levels were higher in the FGR-PN than in the sham-GN group ($P < 0.05$). The FGR-PN group had higher blood glucose than the sham-PN group ($P < 0.05$) and the FGR-GN group ($P < 0.05$) (Figure 2f).

**Pancreatic insulin-positive and total area**

There were no between-group differences for the insulin-positive area, total pancreatic area, or in the ratio of insulin-positive to total pancreatic areas (Figure 3).

**Islet morphology and fibrosis**

Severely dysmorphic islets of Langerhans were evident in male FGR-GN rats. In contrast, slightly dysmorphic islets were observed in female FGR-GN and male FGR-PN rats (Figure 4a–c).

The islets were structurally normal in female FGR-PN rats and in all sham groups for both sexes (Figure 4d–h). Markedly fibrotic tissue was evident only in male FGR-GN rats (Figure 5a).

**Blood biochemistry**

In male rats, FGR-GN rats had a higher average TG level than their sham-PN ($P < 0.01$) and FGR-PN ($P < 0.05$) same-sex equivalents (Figure 6a). HDL levels were higher in FGR-GN than in FGR-PN rats (Figure 6e, $P < 0.05$). There were no significant between-group differences in TCHO levels (Figure 6c). Among female rats, average TG levels were higher for FGR-GN than FGR-PN ($P < 0.05$, Figure 6b). There were no significant between-group differences for TCHO or HDL (Figure 6d, 6f).

**Proteomics**

The proteins extracted from the pancreas lysates of 36-week-old rats were subjected to proteomics and annotation analysis to reveal target molecules associated with mechanisms of histopathological abnormalities and to comprehensively understand the alteration of protein expression in the pancreas.

Proteins classified as b-I and d-I were affected by FGR regardless of nutritional condition (Tables S1 and S2). Proteins classified as b-II and d-II were affected by FGR only under good nutritional conditions (Tables S3 and S4), whereas those classified as b-III and d-III were affected by FGR under poor nutritional conditions (Tables S5 and S6). Similarly, proteins classified as c-I and e-I were affected by the differences in the nutritional conditions regardless of being in FGR or sham group (Tables S7, S8). Proteins classified as c-II, c-III, e-II, and e-III were also affected by the differences in nutritional conditions. The proteins in c-II and e-II were observed only in FGR offspring (Tables S9 and S10), whereas proteins in c-III and e-III were observed only in sham offspring (Tables S11 and S12).

**Functional analysis**

We analyzed the protein interaction networks using functional annotation analysis to

enhance our understanding of their functional associations (Figure 8). This network features molecular interactions among proteins in microtubule/cytoskeleton-related and cell adhesion-related clusters, as shown in the annotation analysis of the b-I profile of male offspring (Figure 8a).

Similarly, molecular interactions among proteins in the cell adhesion-related cluster are shown in annotation analysis of the b-II profile from male offspring (Figure 8b). The figures depict the close relationships of some proteins (e.g., Prkaca, Map2k1, Rap1b, Prkacb, and Gnai2) (Figure 8a, yellow nodes). These proteins categorized into similar ontologies can be further grouped. For example, Prkaca (Figure 8a, yellow node) and Dync1i2 (Figure 8a, red node) were categorized into different groups by STRING and MCL clustering. Furthermore, expression levels of these proteins are presented in Figure 9

**Blood pressure**

Blood pressure was measured at 8, 16, 24, and 35 weeks. BP were similar across the groups (Table S25).

**【Material and methods】**

**Fetal growth restriction rat model**

On day 17 of gestation, pregnant rats were anesthetized with 2.5% isoflurane and ACs 0.4 mm in diameter were attached to ovarian and uterine arteries bilaterally, while five sham mothers underwent laparotomy and gently handled the uterine horns. On postnatal day4, pups were assigned to either the good nutrition (GN) groups with 5 pups/ dam or poor nutrition (PN) groups with 15 pups/dam. After weaning, all pups were fed regular chow food ad libitum.

**【Discussion】**

We evaluated the effects of FGR, followed by an early catch-up growth period, on islet morphology and metabolic functions using the novel FGR model in male and female rats. FGR led to the development of glucose intolerance. Male FGR-GN rats demonstrated dysmorphic and fibrotic pancreatic islets and dyslipidemia. A comprehensive and functional analysis of proteins expressed in pancreas showed that FGR and nutrition status altered extracellular-related protein expression. Male FGR rats had dysregulated expression of proteins related to microtubule/cytoskeleton and cell adhesion. In FGR rats, having GN severely aggravated the expression of proteins associated with cell adhesion.

FGR, followed by GN immediately after birth until weaning, caused dysmorphic and fibrotic islets, leading to glucose intolerance and dyslipidemia in male offspring.

Our results confirmed the existence of a critical period for nutrition intervention in FGR rats. Although introducing normal chow after weaning did not normalize FGR-PN

rats' glucose tolerance, the group's islet morphology was unaffected. Thus, balanced nutrition that does not lead to excessive weight gain before weaning may protect islets from fibrosis and metabolic dysfunction later in life, particularly in male offspring.

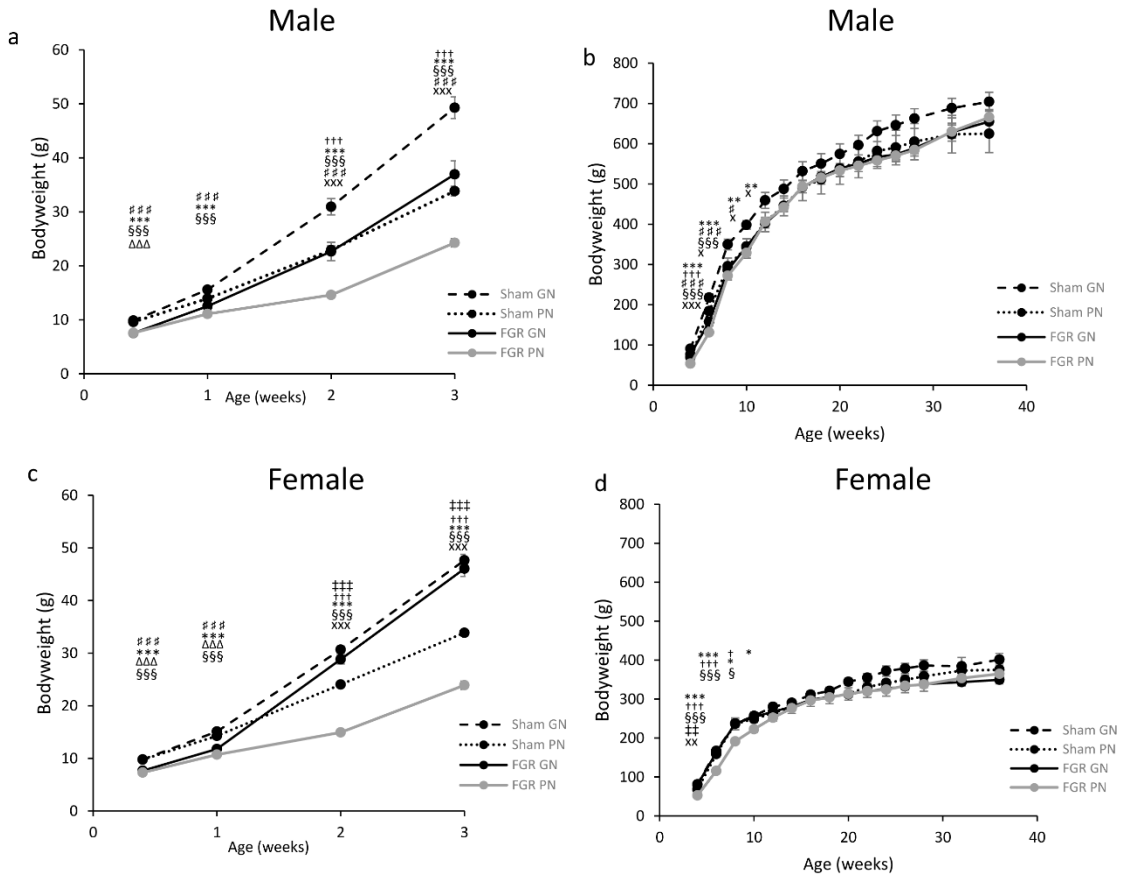


**Figure 1**

**Effects of fetal growth restriction and rapid catch-up growth on body weight.**

(a) Average body weights of male pups from P4 until 3 weeks (weaning on P21), (b) average body weights of male offspring from 4–36 weeks. The FGR groups had significantly lower body weights than the sham groups $P < 0.001$. At 2 weeks, body weight was significantly higher in the FGR-GN group than in the FGR-PN group and in the sham-GN group than in the sham-PN group, $P < 0.001$. At 12 weeks, there was no differences among all groups.

Number of male pups from P4 until weaning: sham-GN: n = 10 (2 groups of 5 pups), sham-PN: n = 7, FGR-PN: n = 8, and FGR-GN: n = 10 (2 groups of 5 pups).

After weaning the number of GN pups reduced to unify with PN groups as shown below:

Number of male offspring from 4–36 weeks: sham-GN: n = 7, sham-PN: n = 7, FGR-PN: n = 8, and FGR-GN: n = 7.

(c) Average body weights of female pups from P4 until 3 weeks (weaning on P 21), (d) average body weights of female offspring from 4–36 weeks. At 2 weeks, body weight was significantly higher in the FGR-GN group than in the FGR-PN and sham-PN groups, $P < 0.001$. Also, the sham-GN group had a significantly higher body weight than the sham-PN group, $P < 0.001$. At 12 weeks, there was no differences among all groups.

Number of female offspring from P4 until weaning (P21): sham-GN: n = 10 (2 groups of 5 pups), sham-PN: n = 8, FGR-PN: n = 7, and FGR-GN: n = 10 (2 groups of 5 pups).

After weaning the number of GN pups reduced to unify with PN groups as shown below:

Number of female offspring from 4-36 weeks: sham-GN: n = 8, sham- PN: n = 8, FGR-PN: n = 7, and FGR-GN: n = 8

FGR-GN vs. sham-PN: ‡ $P < 0.05$, ‡‡ $P < 0.01$, ‡‡‡ $P < 0.001$; FGR-GN vs. FGR-PN: † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$; sham-GN vs. FGR-GN: ♯ $P < 0.05$, ♯♯ $P < 0.01$, ♯♯♯ $P < 0.001$; sham-GN vs. FGR-PN: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; sham-PN vs. FGR-GN: Δ $P < 0.05$, ΔΔ $P < 0.01$, ΔΔΔ $P < 0.001$; sham-PN vs. FGR-PN: § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$, sham-GN vs. sham-PN: x $P < 0.05$, xx $P < 0.01$, xxx $P < 0.001$

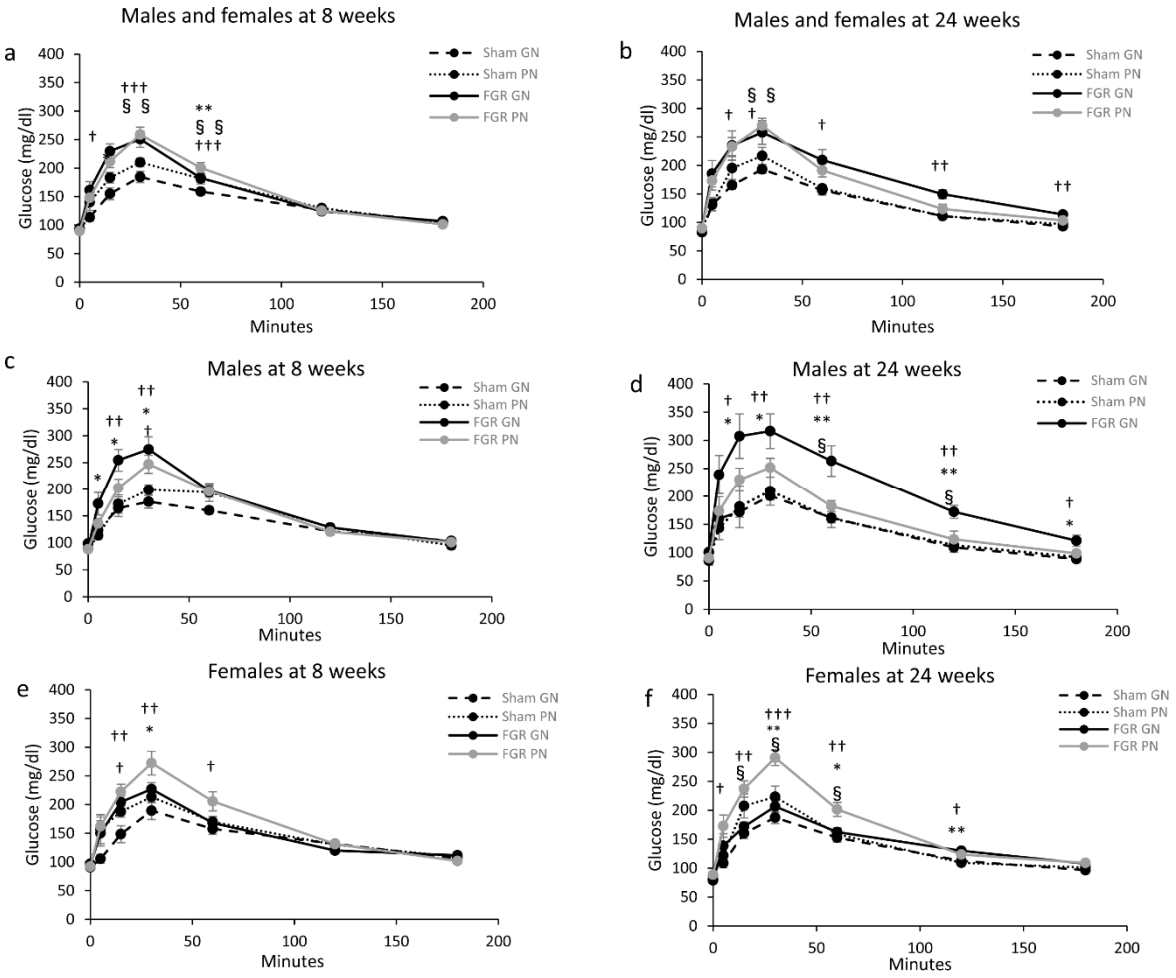


**Figure 2**
**Intraperitoneal glucose tolerance test**
(a) IPGTT males and females (not disaggregated) at 8 weeks. (b) IPGTT males and females (not disaggregated) at 24 weeks. (c) IPGTT males at 8 weeks. (d) IPGTT males at 24 weeks. (e) IPGTT females at 8 weeks. (f) IPGTT females at 24 weeks. Catch-up growth following FGR caused impaired glucose tolerance on the IPGTT after overnight fasting and glucose loading.
FGR-PN vs. sham-PN: * $P < 0.05$, ** $P < 0.01$; FGR-PN vs. sham-GN: § $P < 0.05$, §§ $P < 0.01$; FGR-GN vs. sham-GN: † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$
The number of male offspring: sham-GN: n = 7, sham-PN: n = 7, FGR-PN: n = 8, and FGR-GN: n = 7. Number of female offspring: sham-GN: n = 8, sham-PN: n = 8, FGR-PN: n = 7, and FGR-GN: n = 8
FGR: fetal growth restriction, IPGTT: intraperitoneal glucose tolerance test, GN: good nutrition, PN: poor nutrition.

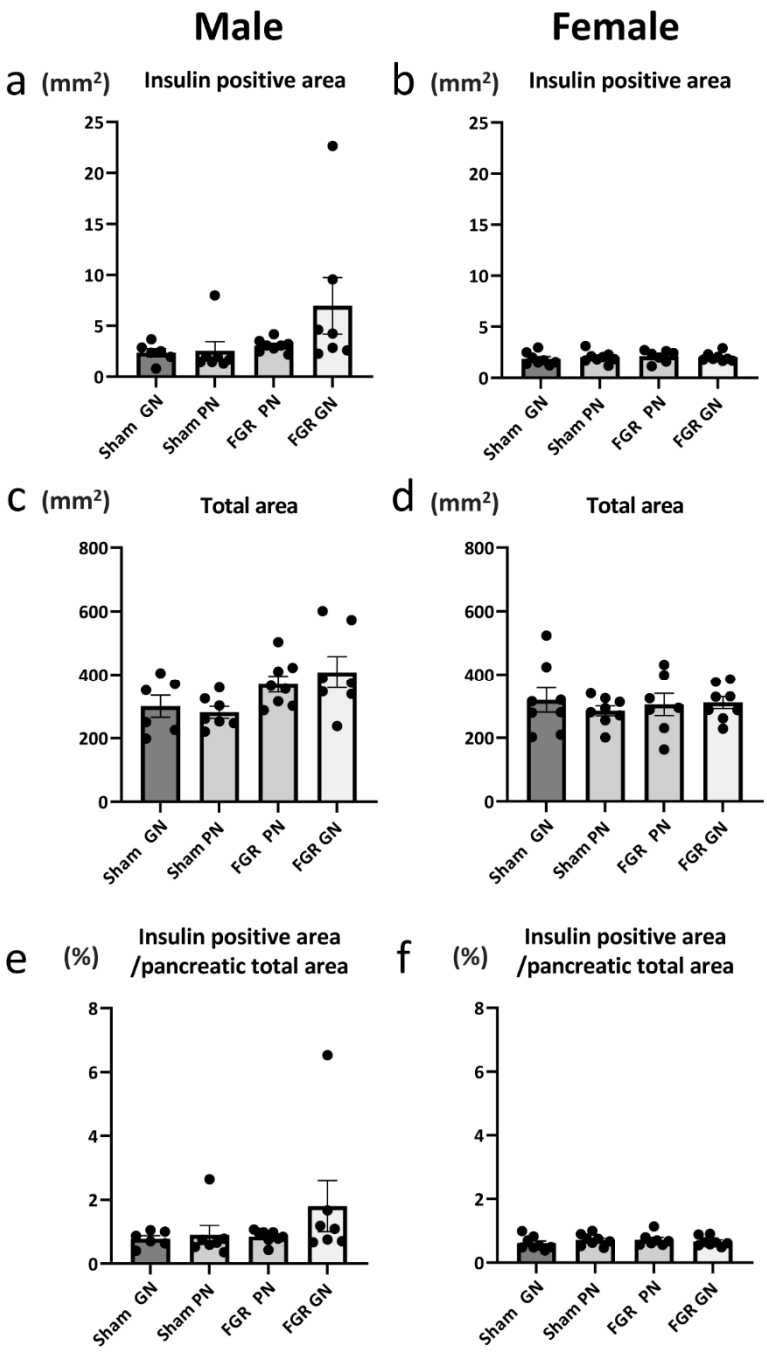


**Figure 3**

**Effects of fetal growth restriction and early catch-up growth on the insulin-positive area.**

In both male and female rats, there were no significant differences in insulin-positive areas (a, b), pancreatic total area (c, d), and the ratio (insulin-positive area to pancreatic total area)(e, f) among the four groups.

The number of male offspring: sham-GN: n = 6, sham-PN: n = 7, FGR-PN: n = 8, and FGR-GN: n = 7. Number of female offspring: sham-GN: n = 8, sham-PN: n = 8, FGR-PN: n = 7, and FGR-GN: n = 8

FGR: fetal growth restriction, GN: good nutrition.

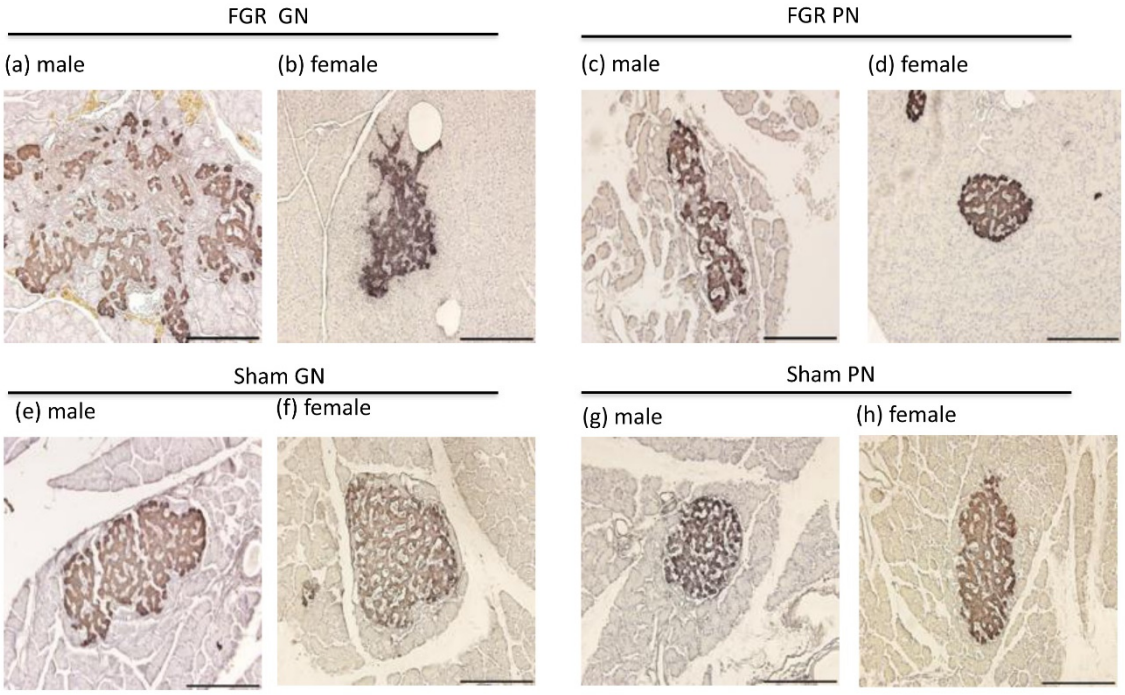


**Figure 4**
Representative photographs of pancreatic islets from 36-week-old male and female rats immunostained with an anti-insulin antibody and counterstained with hematoxylin and eosin at an original magnification of 400×. (a) Islets of male FGR-GN rats exhibited a dysmorphic structure, large size, and scattered islet tissues. (b) Islets of female FGR-GN rats. (c) Islets of male FGR-PN rats showed slight dysmorphisms. The islets appeared normal in female FGR-PN rats (d) and in male and female sham-GN and sham-PN rats (e, f, g, h). Scale bars indicate 200 μm.
The number of male offspring: sham-GN: n = 6, sham-PN: n = 7, FGR-PN: n = 8, and FGR-GN: n = 7. Number of female offspring: sham-GN: n = 7, sham-PN: n = 8, FGR-PN: n = 7, and FGR-GN: n = 8
FGR: fetal growth restriction, GN: good nutrition, PN: poor nutrition.

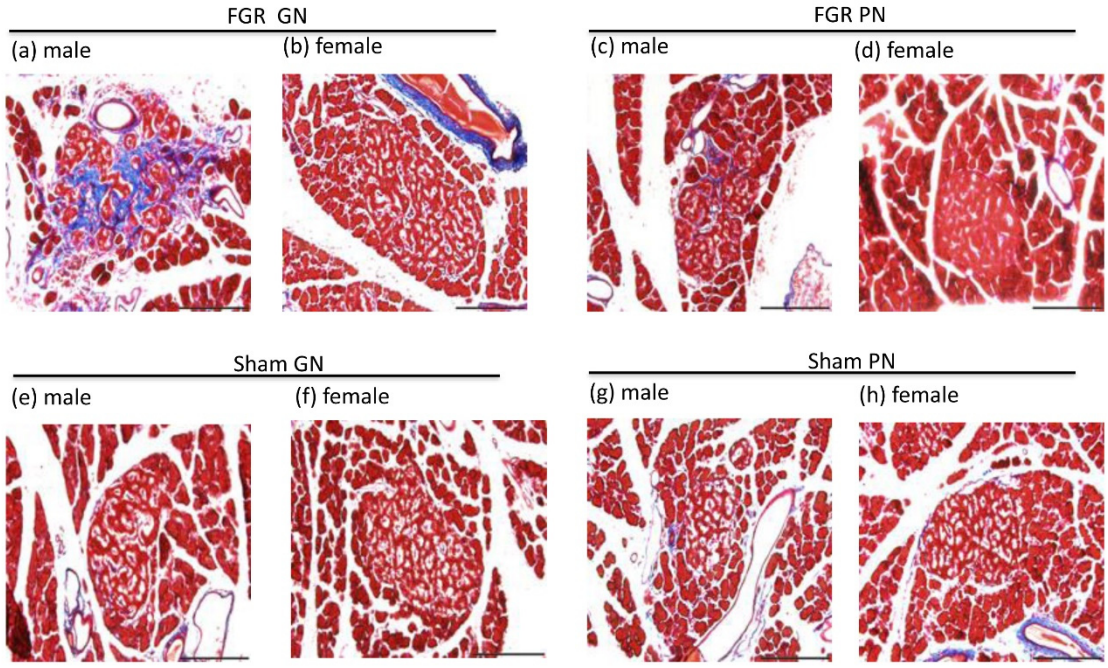


**Figure 5**
Representative photographs of pancreatic islets from 36-week-old male and female rats, Masson's trichrome staining for fibrosis at an original magnification of 400×. Fibrosis was evident in the islets of male FGR-GN rats (a). Fibrosis was not observed in the islets of other groups (b–h). Scale bars indicate 200 μm.
The number of male offspring: sham-GN: n = 6, sham-PN: n = 7, FGR-PN: n = 8, and FGR-GN: n = 7. Number of female offspring: sham-GN: n = 7, sham-PN: n = 8, FGR-PN: n = 7, and FGR-GN: n = 8
FGR: fetal growth restriction, GN: good nutrition.

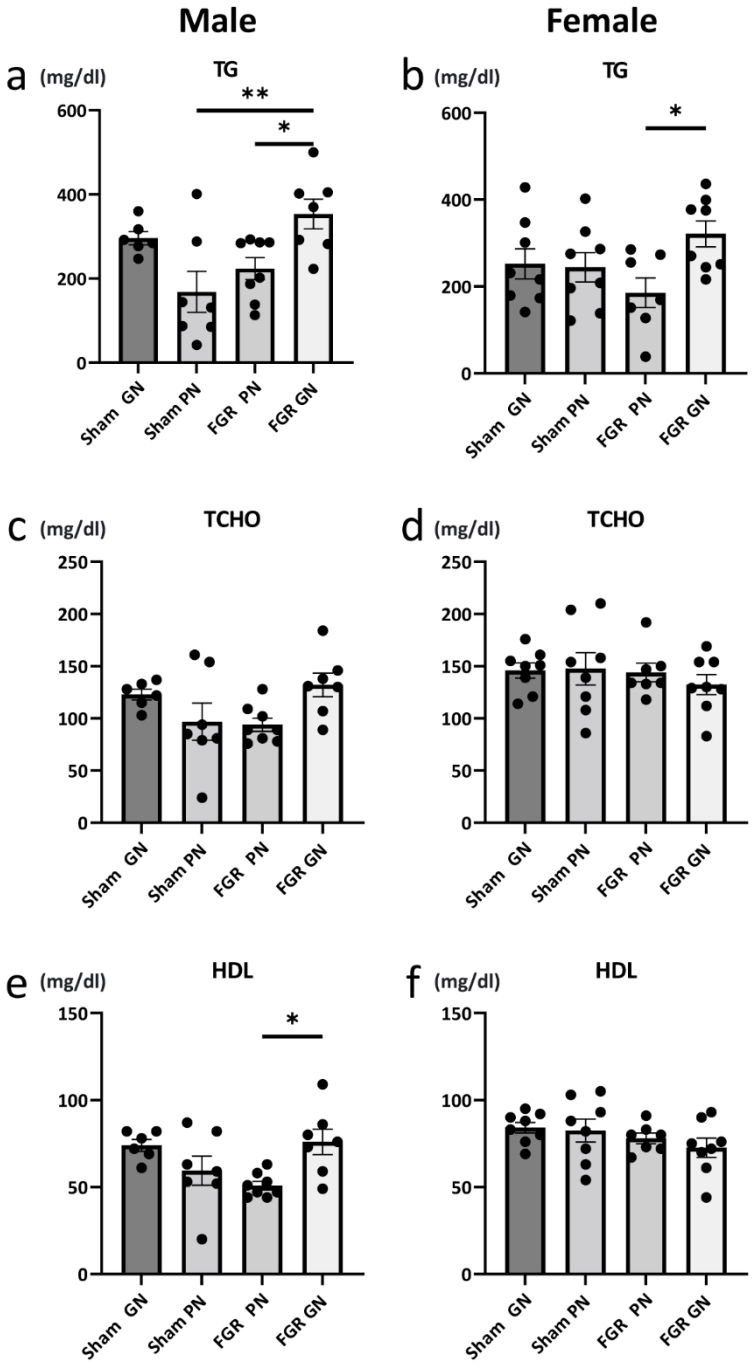


**Figure 6**

**Effects of FGR and early catch-up growth on serum lipid parameters.**

(a) In male rats, the FGR-GN group had a higher TG level than the sham-PN and FGR-PN groups. (b) In female rats, the FGR-GN group had a higher TG level than the FGR-PN group. (e) The average HDL level was higher in the FGR-GN group than in the FGR-PN group. TCHO was similar across all four groups and for both male and female rats (c, d). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

The number of male offspring: sham-GN: n = 6, sham-PN: n = 7, FGR-PN: n = 8, and FGR-GN: n = 7. Number of female offspring: sham-GN: n = 8, sham-PN: n = 8, FGR-PN: n = 7, and FGR-GN: n = 8

FGR: fetal growth restriction, GN: good nutrition, TG: triglyceride, PN: poor nutrition, TCHO: total cholesterol, HDL: high-density lipoprotein cholesterol.

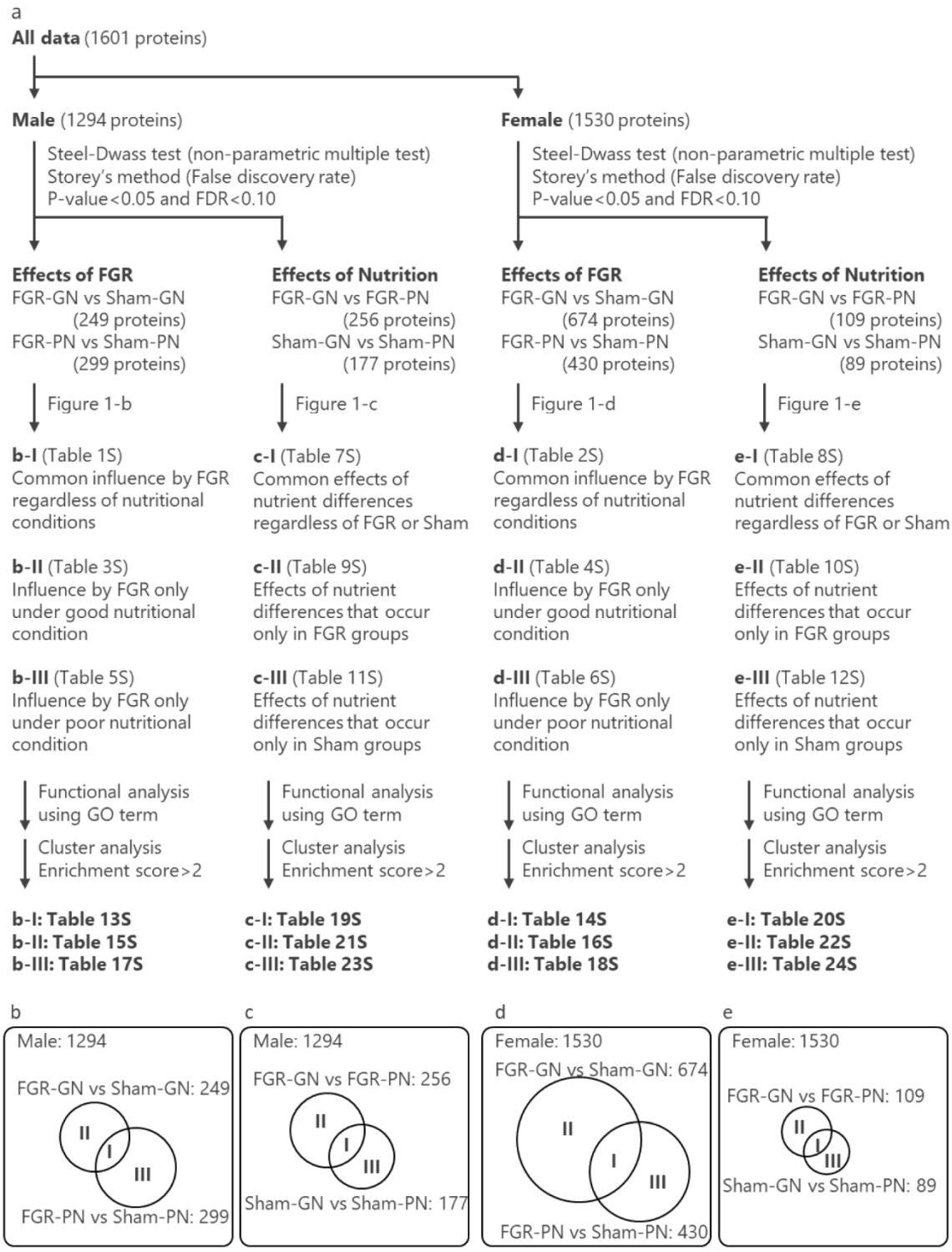


**Figure 7**
**Visualization of the analytical process of proteomics.**
(a) Flowchart of the analytical process and extraction criteria for the pancreatic proteins that were abnormally expressed in each group. (b–e) Venn diagram of the number of extracted proteins. (b, c) Results of the male rats. (d, e) Results of the female rats. (b, d) Results of the comparison between the FGR and sham groups. (c, e) Results of the comparison between the GN and PN groups.
FGR: fetal growth restriction, GN: good nutrition, PN: poor nutrition, GO: gene ontology.

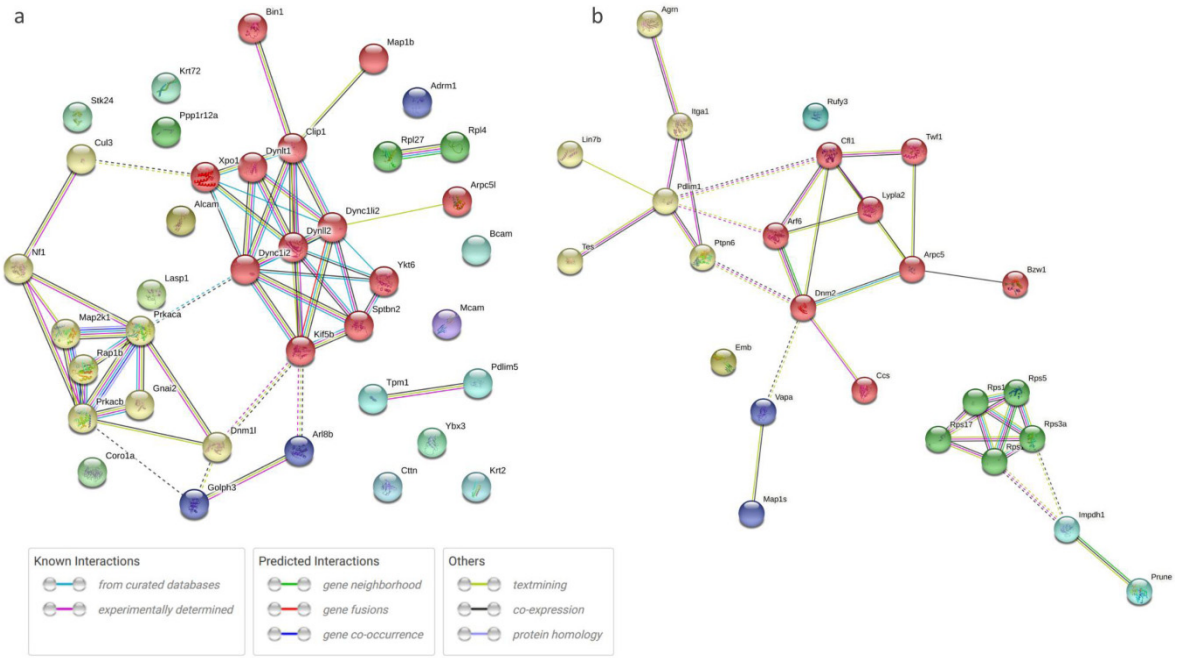


**Figure 8**
**Interaction networks of extracted proteins.**
(a) Interaction of proteins categorized into the microtubule/cytoskeleton-related and cell adhesion-related clusters, as observed in the annotation analysis of the b-I profile (proteins affected by FGR regardless of nutritional conditions). (b) Interaction of proteins categorized into the cell adhesion-related cluster, as observed in the annotation analysis of the b-II profile (proteins affected by FGR-GN only). The figures were generated using the Search Tool for the Retrieval of Interacting Genes/Proteins 11.0 (STRING 11.0), (https://string-db.org/) with MCL clustering (inflation parameter: 3). The minimum required interaction scores were set at (a) 0.400 and (b) 0.150. An interaction was indicated according to eight criteria: neighborhood, gene fusion, co-occurrence, co-expression, experiments, databases, text mining, and homology. Solid lines: specific and meaningful associations like shared function in the MCL cluster. Dashed lines: edges between MCL clusters. FGR: fetal growth restriction, GN: good nutrition, Markov cluster algorithm (MCL), PN: poor nutrition

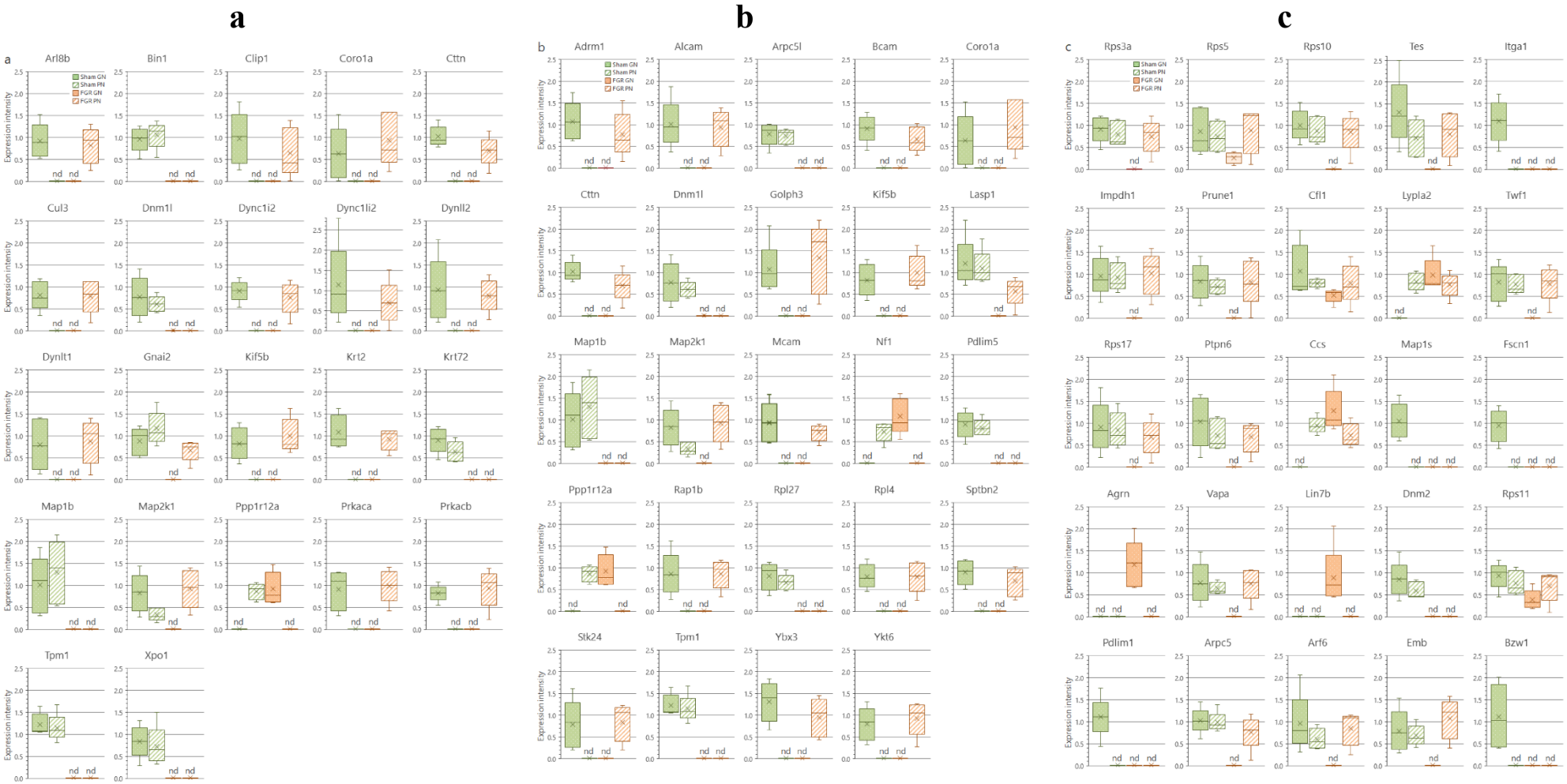


**Figure 9**

**Expression levels of each protein in the clusters found during the functional analysis.**

(a) Expression levels of microtubule/cytoskeleton-related proteins affected by FGR regardless of nutritional conditions (Cluster rank 2 in the b-I profile). (b) Expression levels of cell adhesion-related proteins affected by FGR regardless of nutritional conditions (Cluster rank 3 in the b-I profile). (c) Expression levels of cell adhesion-related proteins affected by FGR only under good nutritional conditions (Cluster rank 2 in the b-II profile). Expression intensity is the relative expression levels to the average value of all proteins detected by liquid chromatography/tandem mass spectrometry (LC/MS/MS) as 1. The detection limit of the LC/MS/MS in terms of relative expression intensity is 0.013. Each box plot means the following groups: Green filled (Left); sham-GN group, Green stripe (Left in the middle); sham-PN group, Orange filled (Right in the middle); FGR-GN group, Orange stripe (Right); FGR-PN group. Abbreviations: FGR: fetal growth restriction, GN: good nutrition, nd: not detected, PN: poor nutrition.

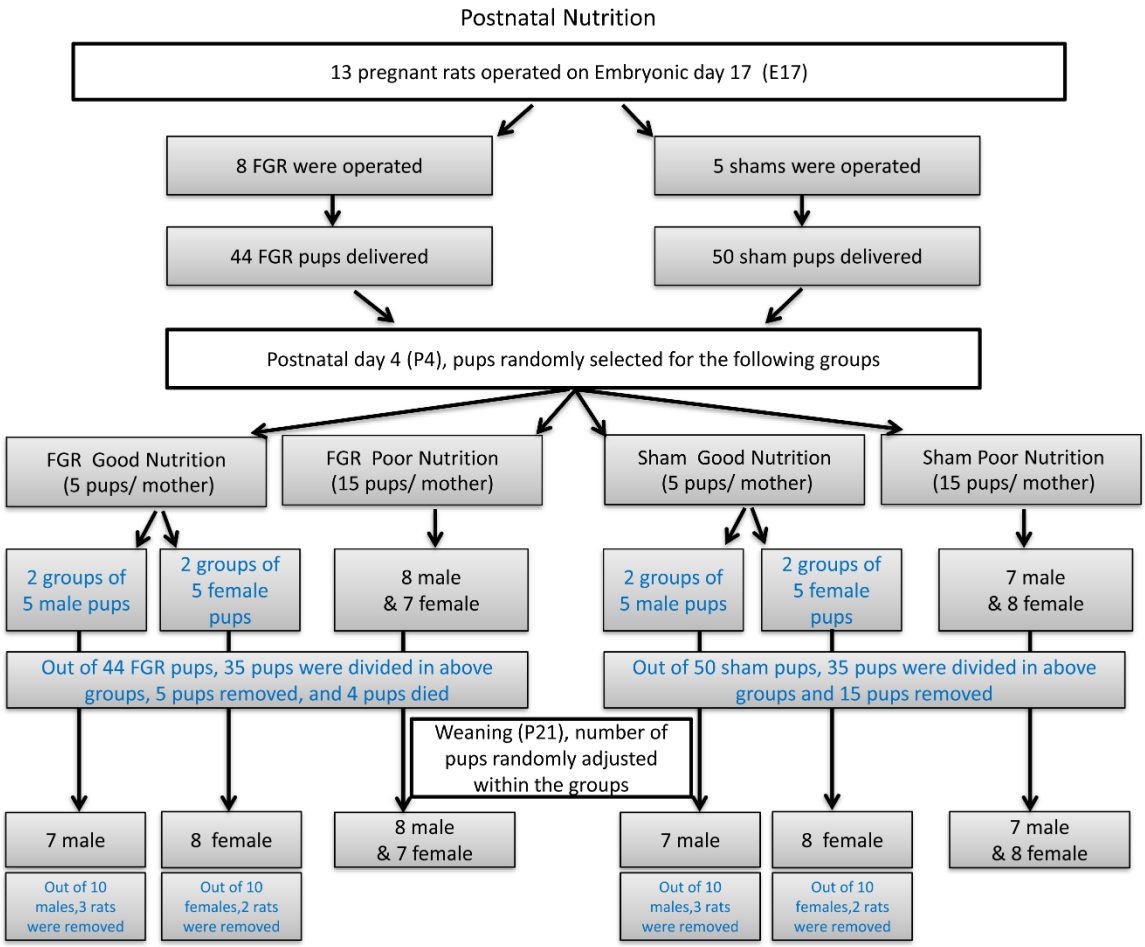


**Figure 10**
Animal participation in the experiments