Notch signaling pathways in the obese mouse

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Abstract

Background: Obesity is a global health problem, affecting people of all ages. The condition is caused by several known and unknown factors from genetics to diet and energy consumption. Recently, studies have shown improved glucose metabolism and loss of body weight when inhibiting Notch 1, a transmembrane receptor involved in numerous developmental processes. However, there is limited research on Notch signaling pathways during the induction of obesity.

Objective: To compare diet-induced obese mice to lean chow-fed control mice with regards to the expression of genes involved in Notch signaling.

Methods: The database Hybrid Mouse Diversity Panel was used for collection of all data. Expression data for genes involved in the canonical pathways of Notch in adipose tissues were chosen, as well as obesity phenotypes (e.g. body fat percentage, insulin levels and liver weight). Mice had been fed either a chow (lean) or an obesity-promoting high fat-high sucrose diet for 8 weeks. Differences in Notch pathway gene expressions between the two dietary groups were investigated and also potential correlations between the components and obesity phenotypes within each group.

Results: All components of the Notch pathway were differently expressed in the diet-induced obese mice compared to the lean controls (p<0.001); eight components were significantly upregulated and only one was downregulated. Fourteen significant correlations were found between Notch pathway components and obesity phenotypes in the obese mice, twelve of them were negative and showed less obesity, lower insulin levels and liver weight.

Conclusion: Diet-induced obesity is associated with a change in gene expression of Notch pathway components. For obese mice, having a higher expression of key Notch pathway components may have a protective effect against obesity when challenged with a high fat-high sucrose diet.

Keywords: Diet; Gene expression; Notch signaling pathway; Obesity
**Abbreviations**

WAT- White adipose tissue  
BAT- Brown adipose tissue  
DLL- Delta like ligand  
JAG- Jagged  
HMDP- Hybrid mouse diversity panel  
HF/HS- High fat/high sucrose
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**Introduction**

Obesity has become a major global health problem, affecting people of all ages. The condition increases the risk of several diseases, including type 2 diabetes, cardiovascular disease and hepatic steatosis. Taken together, obesity and related diseases lead to suffering, an increase in health care costs and premature deaths [1–3]. It is therefore essential to study fundamental mechanisms that can be used to prevent and treat obesity.

Excess adipose tissue is the hallmark of obesity and is the result of several factors, including genetics, diet and energy consumption [3]. Energy homeostasis, the balance between food intake and energy expenditure, is partly regulated by a feedback loop involving the hypothalamus [4]. This regulation is a complex and partially uncharted system, holding several different signaling transduction pathways. Importantly, the regulation of energy homeostasis has been shown to be disrupted in obesity [5]. A breakthrough in obesity research was the discovery of the hormone leptin and its signaling pathways. Leptin is produced by adipocytes and present in serum in direct proportion to the amount of adipose tissue and the concentration is sensed by the hypothalamus regulating feeding behavior [6]. In addition to producing leptin, adipocytes integrate with several regulatory processes involving metabolism, immune responses, blood pressure control, skeletal homeostasis and more. Previously, adipocytes were merely viewed as an energy depot but during the last two decades the scientific interest in adipose tissue metabolism has increased, although many areas still remain unclear [7].

Adipose tissue is generally divided into two different types of cells: 1) white adipose tissue (WAT), with a primary purpose of storing energy and releasing regulatory cytokines and hormones, and 2) brown adipose tissue (BAT) which is essential for thermogenesis (heat production). BAT deposits are primarily found in infants, protecting them from hypothermia [8]. The BAT mass declines with age but is still detectable in adults. Interestingly, BAT is reversely correlated with BMI, which indicate that BAT may also have functions preventing and ameliorating obesity [8,9]. This is further supported by work by Stanford et al. who transplanted BAT to mice and found improved insulin sensitivity, increased glucose uptake and loss of body weight [10].
Recently, so-called beige adipocytes with a gene expression pattern distinct from both WAT and BAT have been described. These adipocytes are thermogenic and located in WAT depots but are in contrast to WAT packed with mitochondria and high levels of uncoupling protein-1 [8]. Beige adipocytes are generated either from beige preadipocytes or converted from WAT. The conversion is regulated by several signaling pathways, as well as by sympathetic nerves and low environmental temperature.

One of the pathways shown to prevent browning of WAT to beige adipocytes is the Notch pathway [11]. Notch is a transmembrane receptor involved in numerous developmental processes through cell-to-cell signaling. There are four known Notch receptors located in different tissues, such as adipose, liver and muscle tissue. The receptors are activated after the binding of the ligands from the delta like ligand (DLL) and Jagged (JAG) families. Upon activation, the Notch intracellular domain, NICD, is released and binds to RBK-Jk, a transcription factor, resulting in the downstream expression of Notch targets, including the Hes and Hey gene families [12]. Of note, in in vitro studies, leptin has been shown to activate and induce expression of Notch pathway components [13].

The inhibition of Notch signaling in mice, through gene deletion or pharmaceuticals, has been shown to increase BAT, improve glucose metabolism and the loss of body mass [11]. In the same study, Notch knockout mice did not gain as much weight as wild type mice despite eating the same high fat diet. In addition, administration of a high fat diet to wild type mice, leads to an up-regulation of Notch receptors [11]. Taken together, these findings suggest that Notch signaling might play an important part in obesity development and prevention. However, there is limited research on Notch signaling pathways during the induction of obesity.

Aim
To investigate the relationship between obesity and gene expression of Notch receptors and their ligands in lean and diet-induced obese mice.
Materials and method

Design and study population

This retrospective study used data from the database Hybrid Mouse Diversity Panel (HMDP). HMDP represents a genetic diversity of mice, consisting of 30 classical and 70 or more recombinant mouse strains [14]. Between two and twelve male mice were used for every strain, and presented as a mean value per strain [15,16]. The data selected from the database was RNA expression data (relative log mRNA expression) of the canonical pathways of Notch from adipose tissue as well as phenotype data (described below). The data were accessed through the laboratory of Kristina Boström, part of the Atherosclerosis Research Unit (ARU) at the University of California Los Angeles (UCLA).

All mice had been fed the same standard chow diet the first eight weeks and were kept in the same environment throughout the study. Control mice were subsequently continued on the chow diet whereas eight weeks of a high fat/high sucrose (HF/HS) diet was used to generate obese mice. At sacrifice all mice were 16 weeks old, and only males were chosen for investigation of tissues and different phenotypical analyses [14–16] The phenotypes chosen for this study were body fat percentage, insulin level in plasma and liver weight, all after 8 weeks of diet. The cutoff significance level for including phenotype correlations was set to p<0.001 after bivariate correlation analysis.

Endpoints

The primary endpoint was the expression of Notch pathway components from adipose tissue of male mice after eight weeks of either the chow or the HF/HS-diet. Secondary endpoints were correlations between expression of the Notch pathway components and phenotypes related to obesity within each dietary group.

Statistical analysis

All data were either normally distributed or log-normally distributed and entered into Microsoft Excel software, version 15.37. To test the significance for Notch pathway components between the two different diets, Student’s T-test (two tailed distribution, two-sample heteroscedastic) was used. A p-value <0.05 was considered as statistical significant and the confidence interval was set to 95%. Pearson’s correlation coefficient and linear
regression tests were used to test the correlation of Notch pathway components and obesity phenotypes.

Five random sample checks were made for every Notch pathway component’s gene expression and obesity phenotype when calculating means for every strain. If a human error was found, the calculation was restarted.

Ethics
The data from the HMDP had already been collected, so no mice have been used specifically for this study. Animal ethics approval for HMDP was obtained by the Institutional Animal Care and Use Committee (IACUC) at UCLA. The database is publically accessible. The results from this essay could be used for further studies to test its transferability to human genomes, and thereby benefit human health.

Results
Gene expression comparisons
We compared the gene expression of Notch pathway components in mice fed a HF/HS diet (obese mice) to the lean control mice. All four Notch receptors had a significantly higher expression in the obese mice (Figure 1).

![Gene expression of Notch receptors after 8 weeks of either a chow (95 strains) or a HF/HS (112 strains) diet. Boxplots showing minimum-maximum values represented by the whiskers as well as the first and third quartile (box).](image-url)
In accordance, Notch ligands were also higher in the HF/HS diet fed mice, with few exceptions. In brief, the DLL 1, 3 and 4 had higher gene expressions in obese mice compared to the controls. The JAG 1- ligand demonstrated the most pronounced change in regulation between chow and the HF/HS diet from being the third highest expressed ligand in control mice, to being the highest in HF/HS diet fed mice. JAG 2 was the only ligand to decrease when comparing obese mice to control mice (Figure 2).

**Figure 2.** Gene expression of Notch ligands after 8 weeks of either a chow (95 strains) or a HF/HS (112 strains) diet. Boxplots showing minimum-maximum values represented by the whiskers as well as the first and third quartile (box).

**Phenotype correlations**

In mice fed with a HF/HS diet, 14 significant correlations were found between Notch pathway components and the obesity phenotypes whereof two of them were positive (Table 1). Notably, no significant correlations were found in the control mice.
Table 1. Correlations between Notch pathway components (after 8 weeks of a HF/HS diet) and obesity phenotypes. P-value shown in parentheses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype</th>
<th>Body fat, %</th>
<th>Insulin, pg/ml</th>
<th>Liver weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOTCH 1</td>
<td></td>
<td>-</td>
<td>-0.57 (4.39E-18)</td>
<td>-0.45 (6.96E-13)</td>
</tr>
<tr>
<td>NOTCH 2</td>
<td>0.27 (5.16E-05)</td>
<td>-</td>
<td>-</td>
<td>0.22 (0.001)</td>
</tr>
<tr>
<td>NOTCH 3</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NOTCH 4</td>
<td></td>
<td>-</td>
<td>-0.34 (1.09E-06)</td>
<td>-0.37 (5.49E-06)</td>
</tr>
<tr>
<td>DLL 1</td>
<td>-0.34 (2.16E-07)</td>
<td>-0.42 (1.32E-09)</td>
<td>-0.34 (1.74E-07)</td>
<td></td>
</tr>
<tr>
<td>DLL 3</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DLL 4</td>
<td></td>
<td>-</td>
<td>-0.36 (1.98E-07)</td>
<td>-0.35 (8.91E-08)</td>
</tr>
<tr>
<td>JAG 1</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>JAG 2</td>
<td>-0.27 (4.32E-05)</td>
<td>-0.36 (6.11E-07)</td>
<td>-0.36 (3.07E-08)</td>
<td></td>
</tr>
</tbody>
</table>

Only DLL 1 and JAG 2 demonstrated significant correlations with all three obesity phenotypes – body fat, insulin level and liver weight. Notch 3, DLL3 and JAG 1 showed no significant correlations to any of the phenotypes. Notch 2 had positive correlations with body fat percentage and liver weight, being the only Notch component to have a significant positive correlation (Table 1). The highest correlation for every phenotype from Table 1 is presented in Figure 3.

Figure 3. Additional charts demonstrating the highest correlation for every phenotype in Table 1. Each dot represents the average values of one mouse strain.
**Discussion**

In this study, we have shown significant changes in gene expression and several correlations with obesity phenotypes. Our findings implicate that Notch signaling pathways are involved in obesity, adding additional knowledge about the complex Notch signaling pathways. Interestingly, no correlations between Notch components and obesity phenotypes were found in mice fed a standard chow diet, yet strong negative correlations were found for the obese mice. This finding may indicate that Notch signaling pathways are activated by a HF/HS diet and therefore are involved in the early development of diet induced obesity.

All four Notch receptors were upregulated in HF/HS diet fed mice. In addition, three of them (Notch 1, 2 and 3) demonstrated strong correlations with the obesity phenotypes. This finding indicates that all Notch receptors respond to a HF/HS diet but may have different functions in metabolism. Similar upregulation of the four receptors were also seen when Pengpeng et al. [11] investigated gene expression of Notch receptors in wild type mice after being fed a high fat diet. Another finding in the same study was that adipocyte-specific knockout mice (Notch 1) showed an improved glucose metabolism and lower weight gain compared to wild type mice after a high fat diet. This is in contrast to our study, where our highest significant correlation was found between Notch 1 and insulin levels in obese mice (-0.57, p-value: 4.39E-18), suggesting that a higher gene expression of Notch 1 could result in lower insulin levels and thereby improve glucose sensitivity in obese mice. A separate Notch 1 knockout study (hepatocyte-specific) was performed by Bernsmeier et al. [17], showing higher insulin levels after 12 weeks of a high fat diet compared to wild type mice. Their result is in agreement with our findings. It further strengthens our result, even though direct comparisons cannot be made since we analysed different tissues and did not use identical diets. For unknown reasons, Notch 2 had positive correlations with body fat percentage and liver weight in our study. These were the two weakest correlations (0.27, p-value 5.16E-05 respectively 0.22, p-value 0.001), which makes the impact of the result more questionable. Further research is warranted to investigate whether this finding represents a true relationship for mice and humans.

The changes in gene expression of Notch ligands were less consistent. DLL 1 was significantly upregulated in obese mice compared to the controls, and was negatively correlated to all three phenotypes. This finding indicates that DLL 1 is activated by a HF/HS diet and may have functions that ameliorate obesity. DLL 3 demonstrated a statistically
significant change in gene expression when comparing obese mice to control mice but the absolute differences between groups were negligible. It is therefore difficult to draw any firm conclusions from this finding. Interestingly, JAG 1, the ligand demonstrating the greatest change between the two diets (upregulated in obese mice), showed no correlation to the obesity phenotypes. This suggests that JAG 1 responds to a HF/HS diet and therefore might be involved in obesity, but perhaps in different ways with different phenotype correlations than those investigated in our study. JAG 2 was the only ligand to have a downregulated gene expression when comparing obese mice to the controls, but was also one of only two ligands to have correlations with all three phenotypes. These negative correlations show a possible protective response from JAG 2, even though the reason for the downregulation of the ligand remain unclear.

Although some of our results deviate from findings in earlier studies [11,18] there are several mechanisms which could can explain the discrepancies. Changes in gene expression are dependent on genetics but also mirror environmental factors, bearing several confounders that may influence the result. Mice from different studies are studied under different laboratory conditions including different diets, feeding schedules, exposure to light and ambient temperature, which makes comparisons more difficult. By having all mice (in the HMDP-database we used) in the same habitat, we minimized the risk of intra-study confounding. Using mice of the same age and sex further minimized such risks, which strengthen our results of how Notch receptors affect obesity. The HMDP-database is an appropriate database to use for this type of association study. The use of both classical and recombined mouse strains provides higher mapping resolution and mapping power [14]. When in control of the mice genetics, it is possible to perform studies where different environmental conditions (e.g. diet) are tested.

Few studies have been performed on Notch pathway components in obesity research. Although this study provides another piece to the puzzle, it cannot be directly transferred to humans but could in future studies be used for comparisons with human genome wide associations studies [19]. Interesting findings could be tested in complementary animal models e.g. pigs or monkeys to further investigate this complex pathway and its possible use for treating obesity. An interesting next step would be to investigate the Notch receptors’ downstream components and their functions, to gain a better understanding of the pathways physiological activity in metabolism.
Limitations

Because of large amounts of data and the time frame provided for this thesis, only phenotype correlations with p-values <0.001 were investigated from the HMDP, even though p<0.05 was considered as statistical significant. Additional physiological information can therefore have been missed. Even insignificant correlations could have been interesting to investigate, perhaps generating a new hypothesis for future studies. Furthermore, associations (i.e. correlations) do not imply causation. To investigate true cause-effect relations more specific tools are required, such as knockout animal models and drugs affecting the pathway described.

No cutoff value for correlations was set in this study, including all correlations with low enough p-values. This resulted in correlations below 0.5 (or above -0.5), which is generally viewed as less meaningful [20].

Only male mice were analysed in this study, therefore additional studies need to be conducted to investigate if the results are extrapolatable to female mice as well.

All data were entered and processed manually, giving rise to the possibility of human error influencing the results. To decrease this risk, random sample checks were made.

Strength of study

The risk of statistical type I and II-errors in this study is considered to be low, because of the high number of mouse strains and total number of mice. Having observed several significant differences, there is a low possibility of this being chance alone.

Conclusion

In this study, diet-induced obesity was associated with an increase in gene expression of Notch pathway components. High expression of key Notch pathway components in obese mice was correlated with less obesity, lower insulin levels and liver weight. This indicates that having a higher expression of key Notch pathway components may have a protective effect against obesity when challenged with a diet high in fat and sucrose. Future studies are required to determine causality.
Acknowledgements

A great thank you to everyone at the laboratory of Kristina Boström, part of the Atherosclerosis Research Unit (ARU) at the University of California Los Angeles (UCLA), for all of your support. Especially Xiuju Wu, for your guidance and patience. A special thank you to my extraordinary sister Dr. Marie Palmnäs, University of Calgary, for great ideas, proof reading and always being there.
References

Dear Dr. X:

I am proud to submit an original retrospective study named “Notch signaling pathways in the obese mouse” by me; Ewa Palmnäs and my co-authors Kristina Boström, MD, PhD and Ole Frøbert, MD, PhD. We hope it will be considered for publication by the Journal of X.

We believe the search for genetic answers to the global obesity health problem is of highest importance and our article is appropriate to publish in your journal because of your common aim to highlight new important findings within genetics.

This manuscript presents new data on the canonical pathways of Notch in mice; their significant changes in gene expression when challenged with a high fat-high sucrose diet and their correlations with several important obesity phenotypes. These findings both add another piece to the puzzle and give rise to further investigations of this complex pathway and its possible use for treating obesity.

Our article is unpublished and is only submitted for publishing consideration at your journal. We have no conflicts of interest to declare. No mice have been used in this study but it is based on a mouse database which has been approved by the Institutional animal and use committee at University of California, Los Angeles.

Thank you for your time!

Sincerely,

Ewa Palmnäs, Bachelor of Medical Science
School of Health and Medical Sciences, Örebro University
Dina gener påverkar din övervikt!


Vi har gått igenom information från tidigare musundersökningar, där möss antingen har fått en normal kost eller en kost med mycket fett och socker. Vi har tittat på proteiner som tillhör något som kallas Notch-gruppen. Denna grupp är viktig för utvecklingen av många delar av kroppen, till exempel vårt fettlager.

De möss som fick för mycket fett och socker hade fler utav Notch-proteiner än de möss som fick en normal kost. Bland alla feta möss så såg man att de som hade fler Notch-proteiner var lite mindre tjocka och mådde bättre än de som hade färre proteiner. Det var alltså skyddande mot fetma att ha fler Notch-proteiner. Kanske kommer vi att kunna använda denna kunskap för att behandla övervikt i framtiden!
Ethical consideration

This study does not include use of animals, but rely on data collected from previous mouse studies. These particular studies have been approved by Institutional Animal Care and Use Committee (IACUC) at University of California Los Angeles.

When using animals for research, you should always weigh their potential suffering to how beneficial the results are to increase scientific knowledge. If not careful considerations are done before a study, for example to choose the best suited species for the study there is a risk of using animals in vain which is not ethically defendable. To minimize the suffering, precautions should be made to reduce physical and psychological pain as well as use as few animals as needed. No unnecessary duplications of animal studies should be made, so a thorough search of what has been studied before should always be made before initiating an animal study.

In this case, the animal study was approved, and to use it for this study which it was not originally made for further strengthens the ethics both for conducting the animal study and this separate study. It increases the scientific knowledge by exploring the database additionally.

Obesity has become a severe condition, affecting millions of people. It is of high importance to conduct research about its origin and possible treatment. The results from this study could be used for further studies to test its transferability to human genomes, and thereby benefit human health.