# Heliyon

# An ameloblastin C-terminus variant is present in human adipose tissue

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## Abstract

**Objective:** Transcriptional regulatory elements in the ameloblastin (AMBN) promoter indicate that adipogenesis may influence its expression. The objective here was to investigate if AMBN is expressed in adipose tissue, and have a role during differentiation of adipocytes.

**Design:** *AMBN* expression was examined in adipose tissue and adipocytes by real-time PCR and ELISA. Distribution of ameloblastin was investigated by immunofluorescence in sections of human subcutaneous adipose tissue. The effect of recombinant proteins resembling AMBN and its processed products on proliferation of primary human pre-adipocytes and murine 3T3-L1 cell lines was measured by [$^3$H]-thymidine incorporation. The effect on adipocyte differentiation was evaluated by the expression profile of the adipogenic markers PPARγ and leptin, and the content of lipids droplets (Oil-Red-O staining).

**Results:** AMBN was found to be expressed in human adipose tissue, human primary adipocytes, and in 3T3-L1 cells. The C-terminus of the AMBN protein and a 45 bp shorter splice variant was identified in human subcutaneous adipose tissue. The expression of AMBN was found to increase four-fold during

differentiation of 3T3-L1 cells. Administration of recombinant AMBN reduced the proliferation, and enhanced the expression of PPARγ and leptin in 3T3-L1 and human pre-adipocytes, respectively.

**Conclusions:** The AMBN C-terminus variant was identified in adipocytes. This variant may be encoded from a short splice variant. Increased expression of AMBN during adipogenesis and its effect on adipogenic factors suggests that AMBN also has a role in adipocyte development.

Keywords: Biochemistry, Cell biology, Molecular biology, Physiology

## 1. Introduction

Multipotent mesenchymal progenitor cells may differentiate into mineralizing osteoblast or adipocyte cells (Nombela-Arrieta et al., 2011; Pittenger et al., 1999; Sadie-Van Gijsen et al., 2013). Stromal cells, human mesenchymal stem cells (hMSC), and osteoblasts are all found to express AMBN (Tamburstuen, Reseland et al., 2011). Recombinant AMBN has effects on both cell proliferation as well as differentiation of hMSC (Tamburstuen et al., 2010). Despite markedly different characteristics, the differentiation of both osteoblasts and adipocytes are modulated by peroxisome proliferation activated receptor gamma (PPARγ) (Kawai and Rosen, 2010). PPARγ is a principal regulator of adipogenesis (Kawai and Rosen, 2010; Spiegelman, 1998; Wu et al., 1996; Wu et al., 1995), and this transcription factor controls multiple regulatory domains located upstream of the *AMBN* gene (Tamburstuen, Snead et al., 2011). However, to the author's knowledge, there are no scientific literature investigating the expression and role of AMBN in adipose tissue.

Adipose tissue expresses and secretes several hormones with importance for endocrine functions (Kershaw and Flier, 2004). These hormones are involved in regulation of intake of nutrients (leptin), control of insulin, inflammation (TNFα, IL-6, resistin, adiponectin) (reviewed in (Coelho et al., 2013)), and blood pressure (Fyhrquist et al., 1995). Several of the so-called adipokines, like leptin (Gordeladze et al., 2002; Reseland et al., 2001), adiponectin (Berner et al., 2004) and resistin (Thommesen et al., 2006) are found to be expressed in bone cells, and to have effects on mineralization. AMBN is processed into two temporally differently expressed splice variants in enamel, of which only the shortest variant seem involved in non-mineralizing cells. This short variant encodes AMBN that consist of the C-terminus part (Lee et al., 2003; Ravindranath et al., 2007). During enamel biomineralisation, the long splice variant is expressed and proteolytically processed (Chun et al., 2010; Geng et al., 2015; Iwata et al., 2007) into other N- and C-terminus products (Uchida et al., 1997; Vymetal et al., 2008), of which only the N-terminus is found in finalized enamel (Castiblanco et al., 2015). The

processing of AMBN and its dominating products are likely to determine its effect on cells. The present study suggests that AMBN is expressed in adipocytes as an early variant that include the C-terminus part of the molecule.

## 2. Materials and methods

### 2.1. Recombinant AMBN and AMBN fragments

Recombinant proteins were expressed in and purified from *Escherichia coli* (*E. coli*). A cleavable tobacco virus etch virus (TEV) specific peptide at the 5'end downstream of thioredoxine (TRX) allowed cleavage of rAMBN from the AMBN-TRX fusion protein, and a 6 x poly-Histidine peptide at the 3'end, as described by Wald et al. (Wald et al., 2013). rAMBN protein includes the amino acids (aa) 27−447 (numbering accounting also for the signal peptide). The N-terminus protein includes the region aa 27−222, and the C-terminus protein includes aa 223−447. rAMBN protein used in proliferation experiments was expressed in *E. coli* with TRX at the N-terminus and with a 6 x polyhistidine tag in the C-terminus (Nakamura et al., 2006). Fig. 1 present an overview of the recombinant proteins used and the location of the splice variant within the exon organization.
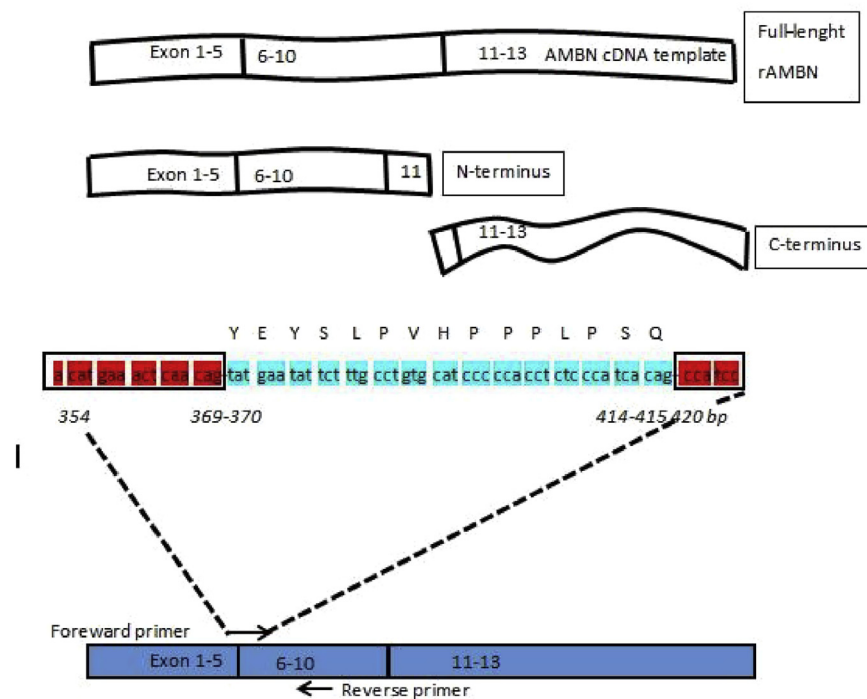


**Fig. 1.** Overview of recombinant proteins used in the experiments: From top rAMBN, N-terminus and C-terminus are shown with exon organization. Below the exon organization are shown with location of the forward primer, bridging the boundary of exon 5/exon 6 of the early AMBN splice isoform I (Lee et al., 2003; Ravindranath et al., 2007).

## 2.2. Cell cultures and tissues

Sections of subcutaneous adipose female tissue were purchased from Zyagen (San Diego, CA, USA). Tissue samples of subcutaneous adipose tissue from healthy donors (aged 36, 50 and 61 years) undergoing mamma-plastic surgeries were isolated by liposuction and RNA isolated. Informed consent were provided by the donors for the storage and collection of adipose tissue (Szoke et al., 2012).

Various cell types were used as positive control in the evaluation of AMBN expression: CD34+ precursor cells were isolated from human bone marrow with approved by the regional ethics committee for medical research and isolated as described previously (Tamburstuen, Reseland et al., 2011). The culturing of human pulp cells (pulpa) (Dominion Pharmakine), differentiation of peripheral blood mononuclear cells into human osteoclast cells (hOC) and the analysis of gene expression were performed as described by Tamburstuen and co-workers (Tamburstuen, Reseland et al., 2011).

Pre-adipocytes (Pt-5001; Lonza, Walkersville, MD, USA) were seeded in a concentration of $5 \times 10^4$ cells/ml in 12 well plates in pre-adipocyte Growth Medium (GM) supplemented with fetal bovine serum, L-glutamine, penicillin/streptomycin (PT-8002; Lonza). Adipogenic differentiation media were prepared from GM supplemented with insulin, dexamethasone, isobutyl-1-methylxanthine (PT-9502; Lonza) (Diff) according to the manufacturer's instructions. As dexamethasone is a strong influencer of PPARγ (Gimble et al., 1996), the effects of AMBN on PPARγ expression was evaluated on cells incubated with adipogenic differentiation media without dexamethasone (Diff - dexa). Cells were grown to 100 % confluence and media was changed to GM, Diff, or Diff-dexa and 0.21 μM rAMBN, N-terminus, or C-terminus, respectively, were administrated. Untreated cells maintained in Diff-dexa or Diff were used as controls for each time-point depending on experimental conditions.

3T3-L1 cells were seeded out in a concentration of $0.5 \times 10^6$ cells/ml in 12 and 48 well plates in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum, L-Glutamine, and penicillin/streptomycin. Cells were differentiated with DMEM supplemented with 20 % fetal bovine serum, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxantine (IMBX), and 2 μM Insulin (Diff). Untreated cells grown in DMEM supplemented with 0.2 μM insulin were used as controls for each time point (the various concentration used here are based on those presented in a review of differentiation protocols of pre-adipocytes (Scott et al., 2011)).

## 2.3. Proliferation assay

3T3-L1 cells were incubated with 2 μg/ml rAMBN. The proliferation of 3T3-L1 cells was measured by [3H]-thymidine incorporation. Sub-confluent cells were

incubated for 24, 48 or 72 hours, and were subsequently pulsed with 1 μCi [$^3$H]-thymidine/well 12 h prior to harvest. Upon harvest, cells were washed with PBS, 5 % Tri-chloric acid (TCA), and the cell contents solubilized in 1 M NaOH. [$^3$H]-thymidine was counted for 3 min in 4 mL scintillation fluid (Lumagel LSC, Groningen, Netherland) in a liquid scintillation counter (Packard 1900 TR, Packard Instruments, Meriden, CT, USA).

## 2.4. mRNA isolation

Adipocytes were washed with cold PBS, lysed, and mRNA isolated using magnetic beads (Oligo(dT)$_{25}$ Dynabeads; Ambion, Life technologies, Oslo, Norway) according to manufacturer's instructions. The RNA concentration was measured on a nanodrop spectrophotometer ND-1000 with software version 3.3.1. Isolated cDNA from human subcutaneous tissue was purchased from Zyagen (San Diego, CA, USA).

## 2.5. Real-time PCR

cDNA was generated from 1 ng mRNA using revertaid First Strand cDNA synthesis kit (Fermentas, Burlington, Ontario, Canada) according to manufacturer's instructions. Real-time PCR (RT-PCR) amplified cDNA for 40 cycles in 20 μl containing SYBRGreen Supermix (Bio-rad, Hercules, CA, USA, Cat.no: 1725272) on the CFX Connect™ system with the Bio-Rad CFX Manager software version 2.1. ΔΔCT values of AMBN, leptin, and PPARγ were normalized to GADPH and beta-actin as reference genes. Primer sequences are given in Table 1.

## 2.6. Immunofluorescence

Formalin-fixed, paraffin-embedded human adipose tissue sections (AMSBio, UK) were deparaffinized through xylene and ethanol series. Epitopes were retrieved with 10 mM citrate buffer pH 6.0 according to the antibody supplier's protocol

**Table 1.** List of primers used in real-time polymerase reactions.

| Gene | Forward | Reverse primer |
| --- | --- | --- |
| h-AMBN | AGAGCACAGTGCATGTCAGC | AAGAACGGCACTGCAAAACT |
| h-LEPTIN | ACAGAAAGTCACCGGTTTGG | GGAAGGCATACTGGTGAGGA |
| h-GADPH | CTC TGC TCC TCC TGT TCG AC | ACGACCAAATCCGTTGACTC |
| h-βeta-actin | TGCTGATCGTATGCAGAAGG | CCCCCAATCCAGACAGAGTA |
| h-PPARγ | TTGAGTGCCGCCGAGTCT | ATTCTGGCCCACCAACTTCGG |
| m-AMBN | AAGAA CAGTGTCACATTTCCTGG | GCGTTTCCAAGAGCCCTGATAAC |
| m-leptin | ATACGGACTGCTTGTGTGAA | AGAAGATCCCAGGGAGGAAA |
| m-βeta-actin | ATATCGTCATCCATGGCGAAC | GCTTCTTTGCAGCTCCTTCGT |
| AMBNΔQ9NP70 | ACATGAAACTCAACAGCCATCC | GCAGCAGCAGACTGGAGAAAA |

(Santa Cruz). All primary antibodies were diluted in 1.5% NGS in PBS, and these included: Anti-AMBN (mouse, C-term epitope (H2), Santa Cruz sc-271012, 1:200), (goat, N-term epitope N-18, Santa Cruz sc-33100, 1:100), and anti-leptin (rabbit, Pierce PA1-052, 1:200). Tissue sections were permeabilised with 0.1% Triton X-100 for 5 min, washed with PBS and blocked with 10% normal goat serum (NGS) and 0.1% Triton X-100 in PBS for 1h, and incubated at 4 °C overnight with the primary antibodies. Sections were then washed three times with PBS before incubated to fluorophore-conjugated secondary antibodies goat anti-mouse Alexa488 and goat anti-rabbit Alexa568 (ThermoFisher), both were diluted 1:400 in 3% NGS in PBS prior to incubation. After 4 h incubation, the sections were washed three times with PBS, incubated with 300 nM DAPI for 30 min, and mounted with Prolong Antifade (ThermoFisher). Sections prepared without primary antibodies were used as controls.

Immunofluorescent staining were examined in a Leica SP8 upright confocal microscope fitted with HyD and PMT detectors, using oil immersion objective 40x HC PL Apo CS2 40x/1.3. Excitation laser lines at 405, 488 and 552nm were used for DAPI, Alexa488 and Alexa568 respectively. Images were produced for publication using FIJI (Schindelin et al., 2012).

## 2.7. Oil Red O staining

Cells were washed with cold phosphate buffered saline (PBS) and fixed with 10 % formaldehyde in PBS in 15 min, before staining with Oil-red-O (3 mg/ml in isopropanol) for 1 h. Remaining Oil Red O solution was then removed and cells were washed with distilled water. Images were taken prior to Oil Red O stain extraction by 100 % isopropyl alcohol. Extracted stain was quantitated at 490 nm. Untreated cells were counter stained with hematoxylin (Merck, Darmstadt, Germany).

## 2.8. Enzyme Linked Immuno Sorbent Assay

Enzyme Linked Immuno Sorbent Assay (ELISA) (Wuhan EIAAB Science Co., LTD. Cat.no UCL-E14174h, East Lake Hi-Tech Development Zone, Wuhan, 430075, China) was used to detect AMBN in 5 x concentrated medium from differentiating adipocytes.

## 3. Results

## 3.1. Adipocytes express AMBN mRNA

Human adipose tissue were found to express AMBN mRNA (Fig. 2A), The AMBN product from human adipose tissue had similar size as the products found in human
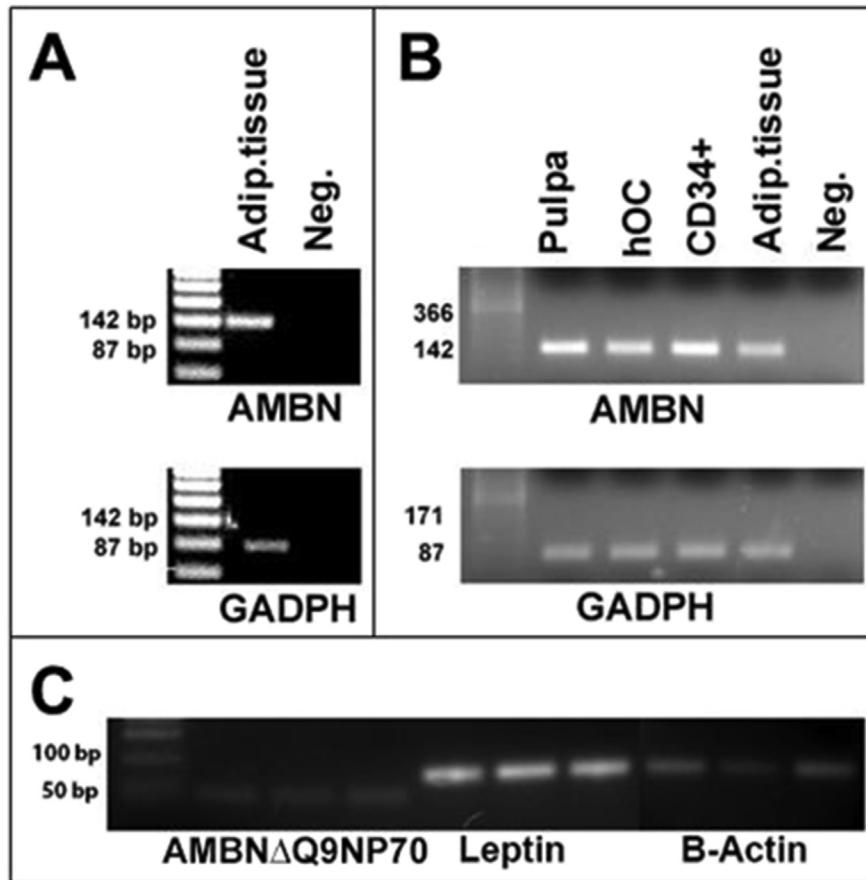
**Fig. 2.** Gene expression of AMBN in adipose tissue: RT-PCR product of AMBN in adipose tissue and glyceraldehydphosphatase (GADPH) (A). RT-PCR product with primers to ameloblastin (AMBN) and GADPH genes in human osteoclast cells (hOC), non-hemapoietic cells (CD34+) and adipose tissue with positive (Pulpa), and negative controls (no template control) (B). RT-PCR product with primers against the exon 5/exon 6 boundary without the 45 bp encoding YEYSLPVHPPPLPSQ in subcutaneous adipose tissue. Noncropped images of the gels are shown in Supplemental Fig. S1.

pulpal cells (Pulp), human osteoclasts (hOC) and in $CD^{34+}$ progenitor cells from human bone marrow (Fig. 2B). The mRNA of a short AMBN splice variant was also identified in human adipose tissue (Fig. 2C).

In differentiating 3T3-L1 cells, the expression of AMBN was found to be increased concomitant to an increase in mRNA expression of PPARγ and leptin. The increase, presented as mean of 3 individual experimental setups, failed to be significant (Fig. 3).

## 3.2. Adipocytes express an AMBN C-terminus variant

Antibodies against the C-terminal AMBN stained the rim of subcellular compartments in mature subcutaneous adipose tissue (Fig. 4B). The merged signals show partly co-localization with the signal against leptin antibodies (Fig. 4C). In contrast,
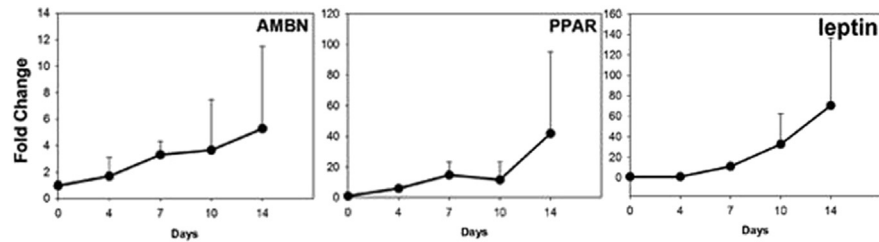
**Fig. 3.** Gene expression of ameloblastin (AMBN), PPARγ, and leptin in 3T3-L1 cells: Shown are the relative levels of AMBN, PPARγ, and leptin gene expression, from left to right. 3T3l-L1 cells were differentiated from confluency and sample at the time point indicated. Messenger RNA isolated and analyzed in real time PCR normalized against β-actin using the ΔΔCt method. All three genes increased with the progress of adipogenic differentiation. Control at 7 days was used to calculate ΔΔCt for leptin at 10 and 14 days shown in the left panel. n = 3.

we were not able to produce images with sufficiently high contrast to identify the signal from the N-terminus antibodies above the background auto fluorescence (Fig. 4K and L, respectively). The expression pattern stained by leptin antibody was in cytosol and around the fixated lipid droplets (Fig. 4D).

## 3.3. AMBN affect cell proliferation and the expression of adipogenic marker and transcription factors

Full-length rAMBN reduced the proliferation of 3T3-L1 cells with 20 % compared to control (P = 0.007) at day 1 (Fig. 5). Only moderate effects of rAMBN (Fig. 6A), N-terminus (Fig. 6B), and C-terminus (Fig. 6C) could be observed on the accumulation of triglyceride droplets, identified with Oil Red O (Fig. 6H) in human pre adipocytes compared to control (Fig. 6D). rAMBN enhanced the expression of PPARγ in human pre-adipocyte cells (P = 0.0004) by 1.5 fold (Fig. 6E). In contrast, the N-terminus part reduced the PPARγ expression (P = 0.009) with 20 % compared to control (Fig. 6G). AMBN enhanced the expression of PPARγ at day 4 in pre-adipocytes during differentiation supported by dexamethasone, but the data failed to be significant (data not shown). AMBN could not be detected in the cell culture media collected during differentiation of human adipocytes within the sensitivity range of the ELISA method used.

## 4. Discussion

We here present evidence for expression and regulation of AMBN in human adipose tissue, human preadipocytes, and the 3T3-L1 cell line, suggesting a putative role for this molecule in adipogenesis. The expression of AMBN was found to be enhanced during differentiation to mature adipocytes, and administration of rAMBN was found to enhance the expression of PPARγ and inhibited the proliferation of 3T3-L1 cells.
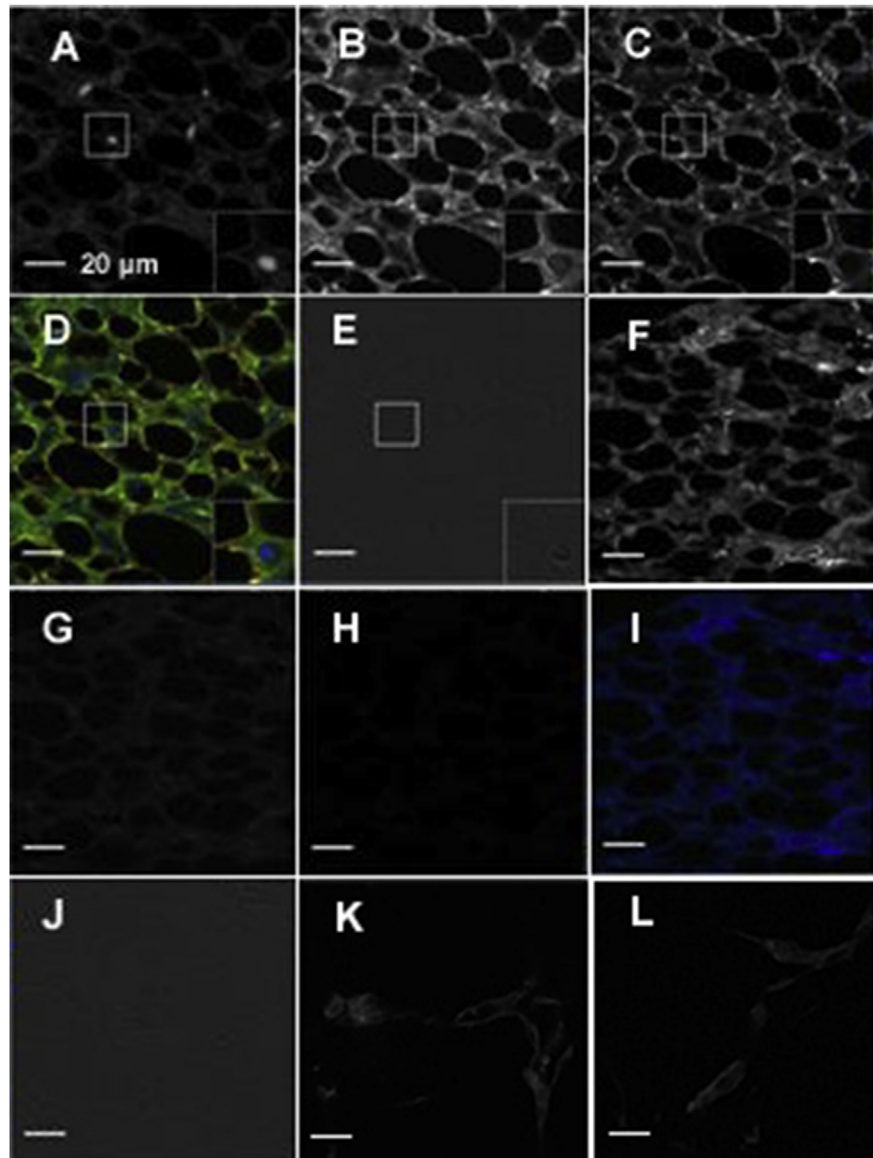
**Fig. 4.** Confocal images of adipose tissue: Sections from subcutaneous human female adipose tissue were scanned for immunofluorescence to reveal expression and subcellular localization of AMBN and leptin. The specificity of the primary antibodies against AMBN C-terminus and leptin was improved by antigen retrieval. The sections were co-stained with DAPI in prolong anti-fade. Upper panels from left to right: DAPI (A), AMBN C-term antibody (B), leptin antibody (C), Merged (D), brigthfield (E). Controls: Dapi (F), secondary antibody (G and H) (as in B and C respectively), merged (I), brigthfield (J), N-terminus (high exposure) (K), and secondary antibody (high exposure related to N-term) (L).

In mature adipose tissue, a short splice variant (−45 bp) of the C-terminus part of AMBN was also detected. The expression of a short splice variant and the C-terminus variant is similar to the expression pattern of AMBN in the early phases of enamel secretion. In the early secretion to maturation phase, ameloblast cells express the same short AMBN splice variant as found here and secrete a smaller 37 kDa
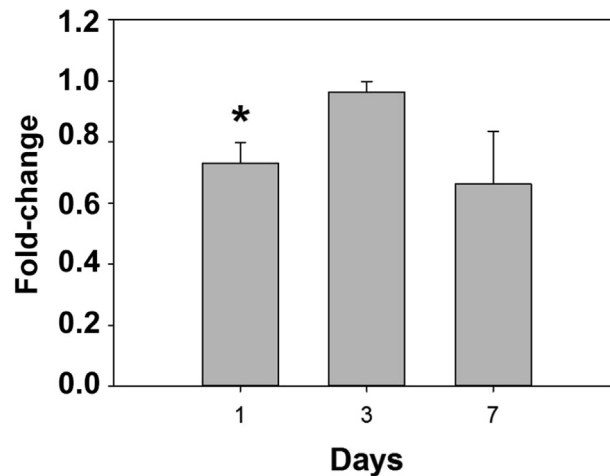
**Fig. 5.** Proliferation of 3T3-L1 cells: Cells proliferation was reduced with AMBN at all time points and significantly at day 1. * indicate $P \leq 0.05$, n = 6.
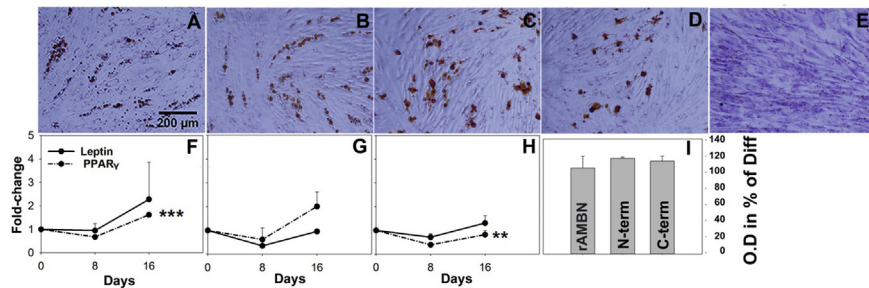


**Fig. 6.** Effects of recombinant AMBN proteins on accumulation of triglyceride and expression of PPARγ and leptin: Human subcutaneous pre-adipocytes incubated with rAMBN (A), C-terminus (B) N-terminus (C), or adipogenic medium without dexamethasone (dexa) (Diff -dexa) (D) and their lipid droplets stained with Oil red O at day 16. Oil red O extracted and quantitated at 490 nm (I) normalized with Diff-dexa. GM is control (E). Gene expression of Leptin and PPARγ in subcutaneous pre-adipocytes administered with rAMBN (F), C-terminus (G) and N-terminus (H). ** indicate $P \leq 0.01$, *** indicate $P \leq 0.001$. n = 3.

AMBN C-terminal protein, while the larger 60−70 kDa size secreted protein is expressed in late maturation, concomitant with expression of the long splice variant (Lee et al., 2003; Ravindranath et al., 2007). It can only be speculated that these AMBN C-terminus variants found in non-mineralizing ameloblast and soft-tissue share similar roles, as both are identified in conjunction with a short splice variant, and recombinant AMBN proteins decrease proliferation of ameloblast as well as adipocytes.

PPARγ expression was enhanced by administration of full-length rAMBN in differentiating adipocytes. Previously, full-length AMBN was found to induce the expression of STAT genes signaling in mesenchymal progenitor cells (Tamburstuen et al., 2010). STAT-1, that was found upregulated, are in the JAK-STAT signaling

pathway involved in regulating lipid turnover in adipocyte cells (Richard and Stephens, 2014). JAK-STAT signaling occurs by cytokine receptors, yet, at present no receptor for AMBN has been identified.

The effect of the recombinant N-terminus part of AMBN seems to have the opposite effect in comparison to full-length rAMBN on pre-adipocytes, reducing the expression of PPARγ. In hMSC, this part of AMBN was identified to enhance the expression of RUNX2 (Stakkestad et al., 2017). We were not able to reliably identify the ABMN N-terminus protein in mature adipocytes, however the presence of N-terminus protein as well as the secretion of various fragments of the AMBN protein from adipose tissue cannot be excluded. It can thus be speculated that the N-terminus products of AMBN promote osteoblast/osteocyte differentiation rather than have a role in adipogenesis. To our knowledge the splice variants of AMBN that exist, only differ in the inclusion or not of the 15 aa YEYSLPVHPPPLPSLQ peptide (MacDougall et al., 2000), and the identification of the short variant in adipose tissue may not account for the lack of N-terminus signal in mature adipocytes.

AMBN C-terminus processing products are also called calcium-binding proteins (Murakami et al., 1997; Vymetal et al., 2008; Yamakoshi et al., 2001). High levels of both extracellular and intracellular $Ca^{2+}$ have been shown to attenuate adipogenesis (Jensen et al., 2004; Shi et al., 2000). Albeit we did not specifically study effect of e.g. AMBN knock out or silencing RNA effects on $Ca^{2+}$ effects in adipogenesis, a potential role for the AMBN C-terminus variant could be to bind and sequester $Ca^{2+}$ and prohibit mineralization of soft tissue.

AMBN gene polymorphisms (Dashash et al., 2011), evolutionary constraints (Delsuc et al., 2015), and knock out of exon 6 (Poulter et al., 2014) has been suggested a cause for non-functional mineralization of enamel leading to amelogenesis imperfecta. If our here proposed role for AMBN in prevention of mineralization proves true, one may surmise that loss of function could lead to e.g. ectopic mineralization of soft tissue.

Proteins that function in contact dependent cell-to-cell signaling are often immobilized in the extra cellular matrix through heparin binding domains. AMBN fits into this category of proteins as it was shown to inhibit proliferation and bind to epithelial cells dependent on heparin binding domains in the C-terminus part (Fukumoto et al., 2004; Fukumoto et al., 2005; Sonoda et al., 2009; X. Zhang et al., 2011). AMBN as well here may remain immobilized to the cell surface of adipocytes, as it was not detected in quantitative amounts in the culture media.

The effects of AMBN in adipocytes seem analogous to effects previously reported in non-mineralizing ameloblasts, e.g. both cell types express a short AMBN splice variant, a translated AMBN C-terminus variant (Lee et al., 2003; Ravindranath

et al., 2007), and administration of rAMBN reduce proliferation (Fukumoto et al., 2004; Fukumoto et al., 2005; Kuramitsu-Fujimoto et al., 2015; Sonoda et al., 2009; Y. B. Zhang et al., 2011). The expression or processing of AMBN in mature adipocytes may thus be similar as in ameloblast cells and the variant that retain the C-terminus end may present a novel function of AMBN in non-mineralizing tissue. The AMBN product(s) have significant biological effects in vitro on both progenitor cell differentiation and adipose cell growth. Although these results suggests that AMBN is expressed and regulated in adipose tissue, the range of samples and conditions tested are to limited to draw conclusion on to what extent AMBN is regulated, how it is regulated and what its function is. To answer these questions whether ABMN has any biological significance in adipose tissue, appropriate knock-in and knock out studies are needed complemented with a more robust set of human adipose tissue samples. However, the findings clearly show that AMBN is expressed in adipocytes, and the shared mesenchymal origin of adipose and bone cells could hint at a common function of this molecule in these tissues.

## 5. Conclusion

Adipocyte tissue expresses the C-terminus part of AMBN, probably encoded as a short splice variant. The increased expression during adipogenesis thus suggests yet another a novel previously undescribed function for AMBN variants in a non-mineralizing tissue.

## Declarations

## Author contribution statement

Øystein Stakkestad, Janne Elin Reseland: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ståle Petter Lyngstadaas: Conceived and designed the experiments.

Catherine Heyward, Tirill Medin, Aina-Mari Lian: Performed the experiments.

Jiri Vondrasek: Contributed reagents, materials, analysis tools or data.

Gita Pezeshki: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

## Funding statement

## Competing interest statement

The authors declare no conflict of interests.

## Additional information

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