In vitro analysis of bone phenotypes in *Col1a1* and *Jagged1* mutant mice using a standardized osteoblast cell-culture system

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Abstract

The mouse is a valuable model organism to study bone biology and to unravel pathological processes in skeletal disorders. *In vivo* methods like X-ray analysis, DXA measurements, pQCT and µCT are available to investigate the bone phenotype of mutant mice. However, the descriptive nature of such methods does not provide insights into the cellular and molecular bases of the observed bone alterations. Thus, first-line investigations might be complemented by cell-culture based methods to characterize the pathological processes at the cellular level independent from systemic influences. By combining well-established assays, we designed a comprehensive test system to investigate the cellular and molecular phenotype of primary calvarial osteoblasts in mutant mice compared to wild-type controls as a first-line phenotyping method. The compilation of 9 different quantifiable assays allows to assess general properties of cell growth and to investigate bone-specific parameters at the functional-, protein- and RNA level in a kinetic fashion throughout a 3-week culture period, thus maximizing the chance to discover and explain new phenotypes in mutant mice. By analyzing mutant mouse lines for *Col1a1* and *Jag1* (Delta-Notch pathway) that both showed clear alterations in several bone related parameters we could demonstrate the usefulness of our cell-culture system to discriminate between primary (*Col1a1*) and secondary effects (*Jag1*) in osteoblasts.

Key words

bone disease, calvarial osteoblasts, nodule formation, Col1a1, Jagged1

Introduction

Although characterized by a hard and rigid nature, bone is a living and versatile organ possessing multiple functions [1]. Given the complex structure and diverse functions of the skeleton, a multitude of acquired or genetically caused diseases of the skeletal system are known. The mouse represents the premier genetic model organism to study human diseases and is a valuable tool for the investigation of bone biology [2]. Various mutant lines with skeletal defects have been developed using either spontaneous or targeted mutagenesis. To date, more than 3400 genotypes with more than 13.300 annotations have been listed in the Mouse Genome Informatics (MGI) database for skeletal phenotypes (http://www.informatics.jax.org). In the Munich ENU (*N*-ethyl-*N*-nitrosourea) mutagenesis screen [3], various mouse mutants with skeletal abnormalities like polydactyly, syndactyly, *Osteogenesis imperfecta*, *Achondrodysplasia*, *Osteoporosis* or rheumatoid arthritis were identified ([4; 5] and unpublished data).

Once a mouse mutant has been established as model for a human bone disease, a comprehensive analysis of its phenotype is important to unravel the underlying physiological and molecular alterations. Phenotypic characterization of skeletal defects can be performed using X-ray analysis, DXA measurements, pQCT and µCT analysis, three point-bending tests or by determination of biochemical markers for bone formation and resorption [6]. Thus, a comprehensive picture of morphological, structural and clinical chemical alterations of the skeletal system in mutant mice can be obtained. However,

such methods cannot assess if the alterations are caused by primary defects or by systemic influences on the bone cells, e.g. by hormonal/metabolic dysregulation (secondary effect). To study pathological processes at the cellular level independent from systemic influences, *in vitro* investigation of primary osteoblasts provides deeper insights into cellular differences of mutant and wild-type animals and can help to elucidate the molecular causes of bone alterations.

We take the advantage of established *in vitro* assays to design a standardized, comprehensive and multifaceted cell-culture system for the comparison of primary calvarial osteoblasts from mutant mice with wild-type controls. This allows us to maximize the potential for the discovery and characterization of new intrinsic pathomechanisms in osteoblasts. In stimulated cultures, the combination of nine different quantitative assays allows to assess general properties of cell growth and to investigate bone-specific parameters at the functional-, protein- and RNA level. The assays are performed at multiple time points throughout a 3-week culture period to receive kinetic results for each parameter (a detailed SOP for the osteoblast cell-culture system is given in supplementary file S1). The system has been validated by the investigation of two mutant lines with clear alterations in bone-related parameters. Compared to wild type, osteoblasts from *Col1a1*^{Aga2} mice, a murine model for *Osteogenesis imperfecta* [5], showed a different behavior in the culture system, which points to a primary defect in these cells as causative for the disease. In contrast, bone alterations of the second mutant line *Jag1*^{Htu}, carrying a missense mutation in the *Jagged1* gene [7], seem to be secondary caused due to systemic effects,

since mutant osteoblasts demonstrate a similar behavior compared to wild-type cells.

Materials and Methods

A comprehensive description of the complete SOP including detailed material and method specification for cell isolation and all assays is provided in supplementary file S1.

Animal housing and handling

Mouse husbandry was conducted under specific pathogen-free (SPF) conditions in compliance with the Federation of European Laboratory Animal Science Associations (FELASA) protocols. All mutants were produced and kept on an inbred C3HeB/FeJ genetic background and maintained by heterozygous intercrosses. Mice received standard rodent nutrition and water *ad libitum*. All animal experiments were performed under the approval of the responsible animal welfare authority.

Workflow

The time frame for a complete analysis of the osteoblast phenotype comprises 5 weeks after birth (Fig. 1). To ensure identical cultivation, cells are prepared in parallel at 4-6 days of age. After 5 days in culture, the cells are stimulated (referred to as T0) and cultivated for 21 days under stimulating conditions. 9 different assays are performed at 5 defined time points to receive kinetic results for each parameter (referred to as measurement days T0 / T3 / T9 / T15 / T21). To avoid differences in the analysis of a single assay, samples are collected and stored until the end of the cultivation period. Thereafter, each assay is

evaluated by analyzing all samples in parallel (referred to as assay analysis). Thus, the simultaneous and uniform analysis of all samples from different measurement days ensured comparable and reliable results. A schedule for sample collection is provided in supplementary file S1.

Cell preparation and cultivation

Osteoblasts are isolated from 4 to 6 day old mice using enzymatic digestion of the calvariae with collagenase [8; 9]. For the culture system, $12.4x10^6$ cells are needed for each group (mut/wt). Briefly, calvariae of decapitated animals are dissected from the skull, released of superfluous tissues, washed in PBS and treated with 4mM EDTA. After predigestion for 7 minutes with 0.1% collagenase IV (Sigma-Aldrich, Germany), the calvariae are incubated twice with 0.1% collagenase solution for 30 minutes to release the cells. After centrifugation the pellets are resuspended in culture medium A (see below). Finally, the cells of each group are pooled and plated in 24-well cell-culture plates ($5x10^4$ cells/well), 6-well cell-culture plates ($2x10^5$ cells/well) and T12.5 cm^2 cell-culture flasks ($2.5x10^5$ cells/flask) according to the scheme provided in supplementary file S1. The isolated cells are cultivated under standard conditions (37°C / 90% humidity / 5% CO_2). The medium is changed twice a week. Three different culture medias are used. Culture medium A for preparation and the initial 5-day pre-culture period as well as for assay A1 (α-MEM supplemented with 10% FCS / 2 mM glutamine / 100 U/ml penicillin / 100 µg/ml streptomycin). Medium B is used for stimulation of the cells (T0) and for maintenance of the stimulation conditions throughout the 3-week culture period (medium A with 10 mM β-

glycerophosphate / 50 µg/ml ascorbic acid). Medium C is needed for assay A2 (medium B with 5% FCS).

Proliferation / Metabolic activity / Protein content / ALP activity (A1)

One 24-well *Lumox*™ plate is used for each measurement day. The sample wells (6 wt, 6 mut) are processed separately. The metabolic activity is assessed by adding *Resazurin* (*CellQuanti-Blue*™ Reagent, Biotrend Chemikalien, Germany) to the culture medium [10]. Thereafter, cells are lysed, transferred into a tube and centrifuged to separate the nucleic fraction (pellet) and the cytosolic fraction (supernatant). Both fractions are stored at -20°C. The supernatant is used for determination of the alkaline phosphatase (ALP) activity and the protein content. The latter is quantified using the BCA Protein Assay Kit (Thermo Scientific, Germany) [11]. The ALP activity of the cells is quantified by 4-nitrophenylphosphate (PNPP) [12]. The pellet is used to determine the proliferation rate of the cultivated cells by their DNA content with a fluorescent dye (*Quant-iT*™ dsDNA Assay Kit, Invitrogen, Germany). Thus, a growth curve for the culture is obtained and the DNA amount can further be used as a reference value to normalize the metabolic activity, protein content and ALP activity in the same sample.

Collagen secretion / Collagen deposition (A2)

One 6-well *Primaria*™ plate is used for each measurement day and the sample wells (3 wt, 3 mut) are processed separately. The culture supernatant is stored at -20°C and the cell layer is fixed with Bouins solution, covered with water and stored at RT. Collagen is determined based on the precipitation of collagen by

Sirius Red [13]. Secreted collagen (supernatant) is precipitated by the *Sircol*™ Dye reagent (Tebu-bio, Germany) and quantified colorimetrically after dissolving the precipitate. Similarly, collagen that became incorporated into the extracellular matrix (ECM) layer is stained with Sirius Red FB3 (Sigma-Aldrich, Germany) and quantified colorimetrically after dissolving the dye from the matrix.

Matrix mineralization (A3)

One 6-well *Primaria*™ plate is used for each measurement day and the sample wells (3 wt, 3 mut) are processed separately. Cells are fixed in 4% PFA, air dried and stored at RT until analysis. Mineralization of the extracellular matrix is determined using Alizarin Red [14].

Nodule quantification (A4)

All 12.5cm^2 cell-culture flasks are used at each measurement day and are analyzed separately (4 wt, 4 mut). Flasks are placed under a stereomicroscope in a special custom-built holding frame to ensure identical positions at each measurement day. Images are taken and the development of nodule-like structures is quantified using ImageJ software.

Gene expression (A5)

One 24-well *Primaria*™ plate is used for each measurement day. Cells of single wells of both biological groups are pooled (1 wt, 1 mut) and cell lysates are stored at -80°C. RNA is isolated, reverse transcribed into cDNA and applied for qPCR, which is performed in 384well plates using the Taq-Man 7900HT device

and *SYBR*®-Green (Applied Biosystems, Germany) for detection. All primers are listed in supplementary file S1. Determination of expression levels is performed by relative quantification according to the $2^{-\Delta\Delta Ct}$ method [15]. For normalization, the C_t values from two housekeeping genes are averaged to obtain a mean internal reference value in each sample.

Immunocytochemistry

For analysis of Pdpn, stimulated cultures were washed with PBS, fixed in 4% PFA for 15min and permeabilized in 0.1% Triton-X100 for 5 minutes. Immunocytochemistry was performed using monoclonal hamster anti-mouse Pdpn (Acris, Germany) as primary and Alexa Fluor 488 goat-anti-hamster IgG (Molecular probes, Germany) as secondary antibody. Specimens were coated with Vectashield mounting medium (Linaris, Germany) containing DAPI for concomitant counterstaining of the nuclei. Samples were observed under an Axioplan2 fluorescent microscope equipped with AxioVision 4.6.3.0 software (Zeiss, Germany).

Statistical analysis

To obtain the number of cells required for the entire culture system, osteoblasts from multiple mice are pooled. Thus, biological replicates are not performed and the n-multiple wells/flasks of each group represent technical replicates, for which a standard error of the mean (SEM) is calculated. In assay A5, single wells of each group are pooled for RNA isolation and therefore, no technical replicates are performed and SEM is not calculated.

Accordingly, the system provide only trends in terms of differences between mutant and wild-type osteoblasts, but the development and course of each investigated parameter during the cultivation period allows the detection of discrepancies between mutant and wild-type cells.

Important considerations

The quality of collagenase IV is important for the yield and vitality of the isolated cells [16]. Also, the individual composition of FCS as the most important supplement for the culture media influences growth and differentiation of the cells critically [17]. Since strong variations in the quality of collagenase IV and composition of FCS can occur between different batches, it is advisable to test different batches for the culture system. We used the number of cells obtained per calvaria as indicator for collagenase IV and the ability of osteoblasts to differentiate (determined by nodule quantification) as criterion for FCS.

Results

Growth and differentiation of the culture

The isolated calvarial cells appeared morphological polygonal/cuboidal (Fig. 2a) and were cultivated under proliferating conditions for 5 days to reach confluence. Differentiation of the cells using β-glycerphosphate and ascorbic acid was morphologically distinguishable by the development of nodule-like structures (Fig. 2b,c), which constantly increased in size throughout the

cultivation period. Standard immunocytochemical analysis for Pdpn, a marker for osteocytes [18], revealed exclusive staining of the three-dimensional nodules in stimulated cell cultures (Fig. 2d). Initial experiments have shown that the ability and degree of differentiation and nodule formation depend on three important conditions. (1) Nodule formation occurred only in cells that were directly isolated from the calvaria (passage P0). Cultures that have been passaged were unable to develop nodules (data not shown). (2) The cell density at the time point of stimulation (T0) considerably influenced the degree of differentiation. The higher the cell density the more nodules were formed (Fig. 2e,f). (3) The quality of the FCS was found to be crucial for differentiation of the culture, whereas number and size of nodules varied between different batches of FCS (data not shown).

Selection of suitable mouse mutants for validation of the culture system

Two mutant lines with obvious bone phenotypes and known mutations have been chosen for the validation of the entire cell-culture system.

The *Col1a1*^{Aga2} mutant line has recently been described as a new mouse model for *Osteogenesis imperfecta* (OI) with a dominant negative mutation in the *Col1a1* gene causing structural alterations of the mutated type I collagen protein [5]. Analysis in the primary screen of the *German Mouse Clinic* (GMC) revealed abnormal gait, significantly reduced body weight and size as well as shortened and bent limbs, toes and a kinky tail. X-ray analysis detected abnormalities in shape and size of many bones and joints. Bone densitometry using DXA analysis showed significantly reduced bone mineral density (BMD), partial BMD (pBMD = BMD measurement w/o head), bone mineral content

(BMC) and bone content (BC) values in mutant mice [5]. pQCT analysis revealed a strong decrease of total, trabecular and cortical BMD in the femoral metaphysis and diaphysis as well as a pronounced reduction of total and cortical/subcortical BMC. Furthermore, the total and cortical area was decreased and the trabecular area increased (Fig. 3a,b). Taken together, *Col1a1*^{Aga2} mice possess a clear bone phenotype and displayed hallmarks of OI symptoms with multiple fractures, scoliosis and an overall decrease in bone mass and density.

The second mutant line, *Jag1*^{Htu} was also identified in the Munich ENU mutagenesis screen based on an abnormal head-shaking phenotype [7]. Mutant mice possess a G → A missense mutation in the *Jag1* gene that causes a nonconservative amino acid substitution in the second epidermal growth factor (EGF)-like repeat of the extracellular domain, a region important for Notch binding. Heterozygous *Jag1*^{Htu} mice were investigated within the GMC (results of all screens can be found at http://www.mouseclinic.de/phenomap/phenomap.html). Importantly, bone densitometry disclosed significantly increased specific BMD (sBMD = BMD related to the body weight) values. Furthermore, pQCT analysis indicated an increase of total and trabecular BMD, total and cortical/subcortical BMC as well as total and cortical area of bone metaphysis and diaphysis in the mutants. The trabecular BMC and trabecular area in the metaphysis showed reduced values (Fig. 3c,d). Taken together, *Jag1*^{Htu} animals depicted a clear bone phenotype with an increase of cortical bone and a higher trabecular bone mineral density compared to controls.

Cellular development of wild type, Col1a1^{Aga2} and Jag1^{Htu} osteoblasts

Given the distinct alterations in their bone parameters, both mutant lines were considered to be suitable candidates for the validation of our primary calvarial osteoblast cell culture system. To determine differences between mutant and control groups we calculated the ratio of the values at each time point for every assay (except the expression analysis). We considered a difference of 20% between mutant and control as a distinct difference (see Supplementary Figure 1).

Proliferation / Metabolic activity / Protein content / ALP activity (A1)

In wild-type cells, proliferation was highest during the initial phase of development and reached a plateau 15 days after stimulation (Fig. 4a). The metabolic activity decreased in the first 10 days and remained at low levels towards the end of the culture period while protein content strongly increased during the first 3-4 days and remained constantly afterwards (Fig. 4b,c). The ALP-activity increased throughout the cultivation period (Fig. 4d). *Col1a1^{Aga2}* osteoblasts possessed a similar behavior for metabolic activity and protein content (Fig. 4b,c). However, *Col1a1^{Aga2}* cells depicted a reduced initial proliferation period followed by a short growth arrest and a second increase towards the end of the culture period (Fig. 4a), and ALP-activity was reduced at all time points (Fig. 4d). In contrast, all 4 parameters were unchanged in the *Jag1^{Htu}* culture (Fig. 4a-d).

Collagen secretion / Collagen deposition (A2)

In wild-type cultures, collagen secretion continuously decreased until day 15 and remained constant thereafter, while matrix collagen deposition increased throughout the 3-week culture period. *Col1a1*^{Aga2} cultures showed clear differences with lower values for both parameters, *Jag1*^{Htu} osteoblasts depicted similar values for secreted and deposited collagen compared to wild-type control at each time point analyzed (Fig. 4e,f).

Matrix mineralization (A3) and Nodule quantification (A4)

The mineralization of the ECM increased in wild-type cultures continuously until day 15 after stimulation and plateaued afterwards. Nodules first appeared after 7 days and increased in size towards the end of the culture period. *Col1a1*^{Aga2} cells showed striking differences in both assays with higher values for ECM mineralization and a significantly reduced nodule formation rate (Fig. 4g,h). Compared to wild-type control *Jag1*^{Htu} osteoblasts possessed an identical behavior for both parameters (Fig. 4g,h).

Gene expression (A5)

The results of the expression analyses for both mutant lines are summarized in Figure 5a. Additionally, the expression of *Col1a1* and *Alpl* is exemplarily graphed below (Fig. 5b,c). In *Col1a1*^{Aga2} cultures, 9 of 13 marker genes varied with an expression difference > 50% in at least one time point or expression differences of 25-50% in at least 3 time points compared to wild-type, while in *Jag1*^{Htu} cultures only 3 marker genes displayed expression differences.

Taken together, calvarial cells from *Col1a1*^{Aga2} mice showed significant differences in almost all parameters analyzed whereas *Jag1*^{Htu} osteoblasts were nearly indistinguishable from wild-type controls.

Discussion

The calvaria as flat bone is formed by intramembranous ossification whereas most of the appendicular and axial skeleton is formed by endochondral ossification [19]. In a recent study [20], osteoblastic cells obtained from different sources (calvaria vs. long bones), from different ages (adult vs. fetal) and different isolation techniques (explant vs. enzymatic) were compared to each other. The authors demonstrated that enzymatic released fetal calvarial cells are most suitable concerning proliferative capacity and osteogenic potential [20]. Therefore, we have chosen enzymatic isolation of bone cells from neonatal calvariae for the cell-culture system. However, the authors also found differences in ALP-activity and mineralization between *in vitro* cell cultures from calvariae and long bones. Therefore, the intramembranous origin of the calvaria should always been taken into account when results are correlated to bone alterations observed in long bones (endochondral). Furthermore, neonatal cell cultures behave differently and contain more immature, rapidly growing cells than cultures from adult bone [9]. Therefore, also the age of the animals has to be considered for the interpretation of results from a mutant line with a late onset of the bone disease.

In our cell culture system, Isolated calvarial cells possessed the typical cuboidal morphology of osteoblasts [9] and the degree of their differentiation strongly correlated with the initial cell density and the FCS used [17, 21].

Nine different assays have been designed to ensure a comprehensive investigation of the osteoblast phenotype. ALP, mineralization and nodule formation are widely used indicators for the osteogenic potential of osteoblast cell cultures [17, 20]. Nodules, which only appeared in stimulated cultures, contained osteocyte-like cells [22] confirmed by immunofluorescence staining of Pdpn [18]. Consequently, the rate of nodule formation is an indicator for the differentiation to a more mature (osteocyte-like) phenotype. Because collagen is the most abundantly expressed protein in the ECM and isolated osteoblasts synthesize primarily type I collagen (* 95 %) [21], we implemented the measurement of collagen secretion and deposition to further assess the osteogenic capacities of mutant and wild-type osteoblasts. Finally, we analyzed 13 bone-relevant marker genes covering all developmental stages of in vitro osteoblast culture by qRT-PCR [23]. All assays have been designed to allow the quantification of parameters in a kinetic way throughout a 3-week culture period, to identify distinct phenotypes.

Validation of the culture system

To validate our cell culture system and to examine the practicability of its SOP, we analyzed two mutant lines with striking bone phenotypes.

Wild-type osteoblasts

The course of cell growth reflects three distinct proliferation periods (exponential growth, postconfluent proliferation and compensatory proliferation) [24]. Given that replication and cell growth are energetically expensive processes, the metabolic activity of the culture is highest at the beginning and decreases during matrix maturation and mineralization when the initial proliferation is restricted to differentiation [24, 25]. In contrast, protein synthesis is low during the initial proliferation phase but increases when the ECM is produced and remains high for matrix maintenance and reorganization.

The osteogenic potential of the culture markedly increases at the end of the growth period, according to the reciprocally and functionally coupled relationship between the decline in proliferative activity and the subsequent induction of genes (e.g., *Spp1*, *Bglap*) associated with matrix maturation and mineralization [23, 25]. Although *Col1a1* mRNA peaked during the proliferative phase, the overall synthesis and incorporation of collagen into the ECM increased during the entire culture period [25]. This time lag between mRNA expression and collagen incorporation is consistent with a previous report demonstrating uncoupling of type I collagen synthesis and collagen accumulation in developing osteoblasts [26]. Taken together, the cells recapitulate the in vitro developmental sequence of proliferation, differentiation and ECM maturation/mineralization [23]. Therefore, the cell culture system is highly suitable to study bone cell characteristics of different mutant mouse lines.

Aga2 osteoblasts

In comparison to wild-type, calvarial cells from *Col1a1*^{Aga2} mice displayed different characteristics of osteoblast development. No obvious differences

where found in the metabolic activity and overall protein synthesis, which is consistent with a report demonstrating that the amount of total protein synthesized by human OI bone cells in culture was unchanged compared to controls although collagen itself was significantly reduced [27]. Similar to our results for *Col1a1*^{Aga2} osteoblasts, a decrease of the maximal in vitro growth rate was described for osteoblasts from OI patients [27, 28]. Astonishingly, matrix mineralization of *Col1a1*^{Aga2} cells as measured by calcium incorporation was higher compared to controls. Recent findings suggest coherence between collagen structure and mineralization pattern [29]. Thus, it is conceivable that the incorporation of mutated Col1a1 chains into the *Col1a1*^{Aga2} matrix might involve alterations of nucleation sites leading to higher calcium incorporations. Indeed, our findings are in correlation to studies that demonstrated higher mineralization in human OI patients [30].

The alterations on the protein and functional level in *Col1a1*^{Aga2} could be confirmed by expression analysis, wherein almost all bone marker genes were altered indicating diminished differentiation and matrix maturation. For example, the downregulation of the ECM protein genes *Bglap*, *Spp1* and *Ibsp* at the end of the cultivation period is in accordance with a disordered osteoblast differentiation [31–33].

The results obtained from the *Col1a1*^{Aga2} culture could be explained by interactions between proliferation, ECM maturation and osteoblast differentiation. The expression of the mutated type I collagen in *Col1a1*^{Aga2} osteoblasts yields in the activation of endoplasmic reticulum stress and induction of apoptosis as well as reduced type I collagen secretion accompanied by structural and functional ECM impairments that also explain

the discrepancy between collagen expression and secretion/deposition [5]. Since a proper ECM is necessary for cellular differentiation and function [19], and a critical role of type I collagen in mediating the expression of a mature osteoblast phenotype has already been proposed [34], the irregular ECM in *Col1a1*^{Aga2} cultures prevent appropriate osteoblast development. Furthermore, proliferation needs to be decreased for differentiation to occur [23, 25]. Therefore, the increased proliferative activity of *Col1a1*^{Aga2} cells towards the end of the culture period might impede or delay the transition between proliferation and differentiation. The reduced proliferation at the beginning and the increased proliferation towards the end of the culture period might be explained by the previously reported link between type I collagen synthesis and cellular proliferation, which claims that destabilized collagen triple helix formation and altered collagen secretion decreases the growth rate [28]. The increased apoptotic rate in *Col1a1*^{Aga2} osteoblasts [5] might further account for the decreased proliferation, whereby the second rise in proliferative activity might be seen as a compensatory mechanism.

Given that the osteoblasts are cultivated under identical conditions independent from systemic influences, the differences between mutant and wild-type cells refer to a primary defect in *Col1a1Aga2* osteoblasts as the cause for the bone phenotype, in which inappropriate collagen synthesis and secretion result in a diminished or at least delayed extracellular matrix maturation and osteoblast differentiation.

Jag1^{Htu} osteoblasts

Jag1^{Htu} mice possess a mutation in the *Jag1* gene [7]. Mutations in human *Jag1*

are responsible for Alagille syndrome (AGS), a pleiotropic developmental disorder characterized by cholestasis due to bile duct paucity, poor lung function, cardiac abnormalities and skeletal alterations such as butterfly vertebrae [35]. In contrast to AGS patients, who suffer from osteopenia due to hepatic osteodystrophy [36], *Jag1*^{Htu} mice had more robust bones. Given that the mutation in *Jag1*^{Htu} resides in the second EGF-like repeat of Jag1, which is important for Notch binding [7], it is conceivable that a failing of Notch activation accounts for the augmented bone phenotype. Previous studies have shown that Notch normally inhibit osteoblastogenesis and impairs osteoblast differentiation [37] and transgenic mice lacking Notch signaling in skeletogenic mesenchyme exhibit increased bone parameters [38]. However, the effect is reversed in older mice and long-term inhibition of Notch results in osteoporotic mice. This phenotype has never been observed in aged *Jag1*^{Htu} mice in vivo. Moreover, except for an upregulation of *Bglap* and *Ibsp* in the gene expression analysis, the in vitro cultivation of osteoblasts under identical conditions without systemic influences revealed an identical behavior and phenotype of *Jag1*^{Htu} osteoblasts compared to wild-type controls. Taken together, this argues against a primary defect with failing Jag1- Notch interaction in osteoblasts
as a cause of the bone phenotype in *Jag1*^{Htu}.

As *Jag1*^{Htu} mice display an increased angular velocity and forward locomotor activity, which might be related to the vestibular defect in these mutants [7], a systemic effect might be responsible for the bone phenotype in these mutants. Previous studies have shown that enhanced locomotor activity in laboratory mice markedly increased BMC, bone mass and density as well as cortical area of long bones and concomitantly enhances bone mechanical properties [39,

40].

Alternatively, a failing interaction between osteoblasts and hematopoietic stem cells (HSC) could also account for the osteopetrotic phenotype of *Jag1*^{Htu}. Studies addressing interactions of those cell types demonstrated recently that Jag1 expression in osteoblasts could induce Notch signaling in HSC and affect osteoclastogenesis [41]. Thus, an inadequate induction of Notch in HSC by the mutated Jagged1 could lead to an improper osteoclastogenesis, yielding in the osteopetrotic phenotype of *Jag1*^{Htu}. This possibility cannot be ruled out by our cell culture system.

Concluding remarks

This cell–cell culture system was designed for a first-line phenotyping of osteoblasts on the cellular level. If distinct differences are apparent, it will be necessary to examine single animal samples to receive biological replicates in the different assays. Furthermore, methods might be extended (e.g., whole-genome transcriptomics) or added (e.g., protein analysis of RANKL and OPG) to further elucidate molecular mechanisms.

The skeletal system is maintained by a precisely regulated interaction between bone formation (osteoblasts) and bone resorption (osteoclasts) in the process of remodeling (homeostasis). Thus, the investigation of osteoblasts allows for estimating the formation process only but alterations in bone resorption and osteoblast–osteoclast interactions cannot be disclosed. Therefore, in vitro analysis of osteoclasts can be implemented to broaden the phenotyping options of the cell culture system. Tartrate-resistant acid phosphatase staining and pit

formation assays could be performed to evaluate osteoclast development and function [42]. To further extend the cellular analysis and unravel possible dysregulations in cell–cell communication, coculture systems can be applied combining, for example, osteoblasts with mesenchymal stem cells, osteoclasts or chondrocytes.

Conflict of interest:

All authors have no conflicts of interest.

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Figure 1

Workflow of the cell culture system.

Figure 2

Osteoblast cell culture. **a** Two days after preparation single calvarial cells are visible. **b,c** Nodule formation in primary calvarial cell cultures. After stimulation with ascorbic acid and β-glycerophosphate, nodule-like structures are formed with morphological appearance of woven bone (b = day 8; c = day 11). **d** ICC for E11 on primary calvarial cultures (day 7). After stimulation, E11 is expressed only within the nodules (green - E11; blue - DAPI counterstain). **e,f** Influence of cell density on nodule formation. In cultures with 5x105 initially plated cells (n = 3) the number (e) as well as the total area (f) of nodules is higher throughout the culture period compared to cultures with initially 2.5x105 plated cells (n = 3). The observed decrease in the number of nodules towards the end of the culture period in cultures with 5x105 plated cells results from the fusion of nodules when they increase in size. Scale bar in a,b = 250 µm; c,d = 50 µm.

Figure 3

pQCT scan of transversal sections of the femoral metaphysis (resolution 70µm). **a,b** pQCT scan of *Col1a1*^{Aga2} femur (17 week old mice). Compared to wild-type control (a), there is a decrease of total, trabecular and cortical BMD as well as total and cortical area in *Col1a1*^{Aga2} (b). **c,d** pQCT scan of Jag1Htu femur (19 week old mice). Compared to wild-type control (c), there is an increase in total and trabecular BMD as well as total and cortical area in *Jag1*^{Htu} (d).

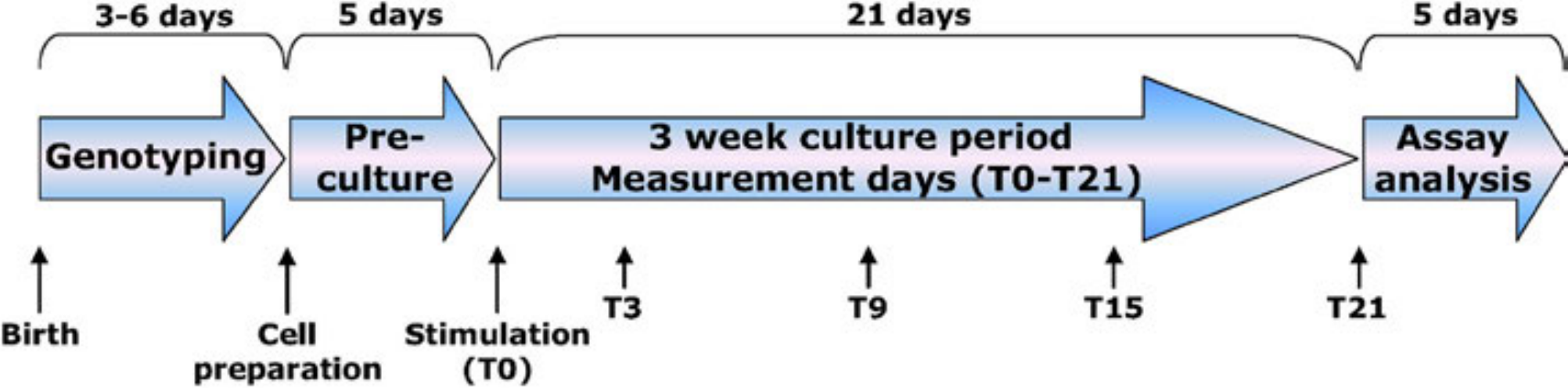
Figure 4

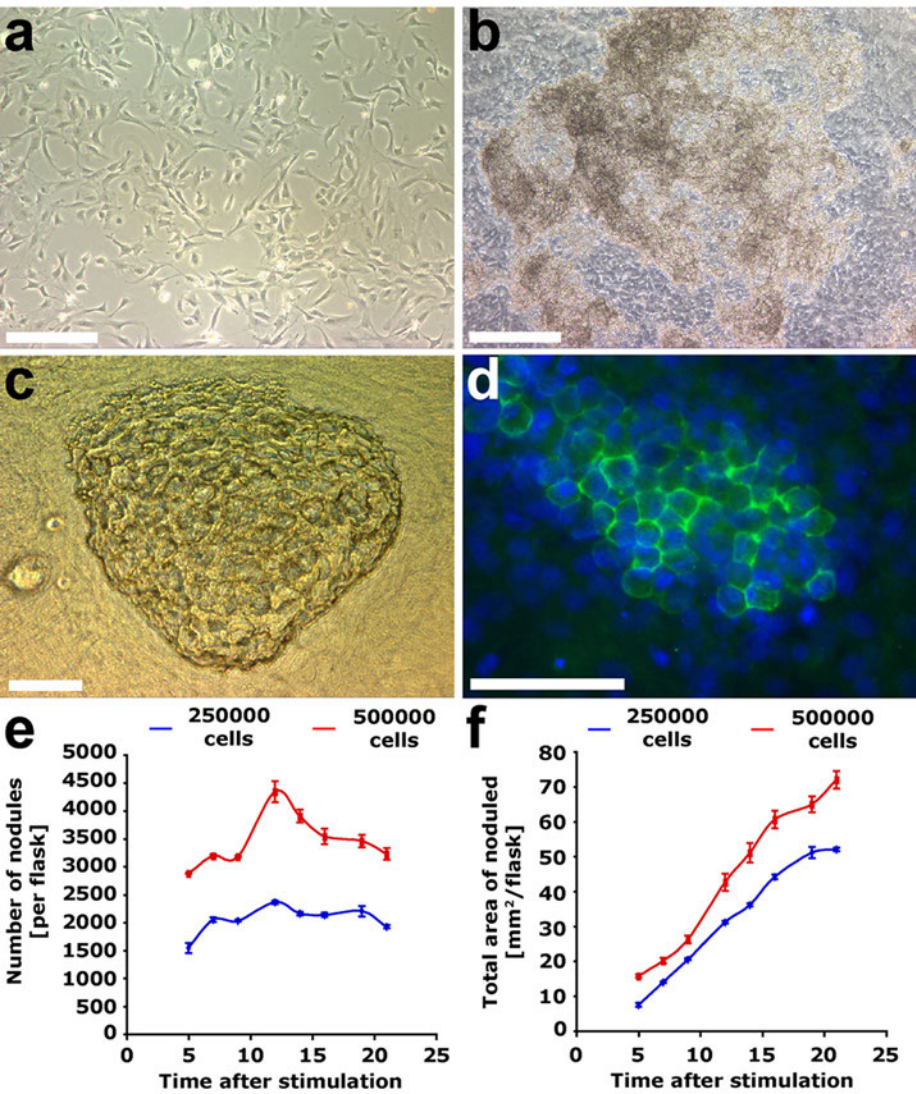
In vitro analysis of primary calvarial osteoblasts from *Col1a1*^{Aga2} and *Jag1*^{Htu} mice. The analysis was performed according to the developed SOP and the results of both studies are displayed with diagrams for each assay. The error bars represent the SEM values calculated from the technical replicates (number of wells per assay, see SOP). The y-axis indicates the assay and the x-axis the day of analysis (for h, deviating from the SOP, measurements were performed at different days for *Col1a1*^{Aga2} and *Jag1*^{Htu} due to technical reasons). While in calvarial cultures of *Col1a1*^{Aga2} mice almost all parameters differ considerably compared to wild-type control cells, no obvious differences can be observed for calvarial cultures of *Jag1*^{Htu} mice.

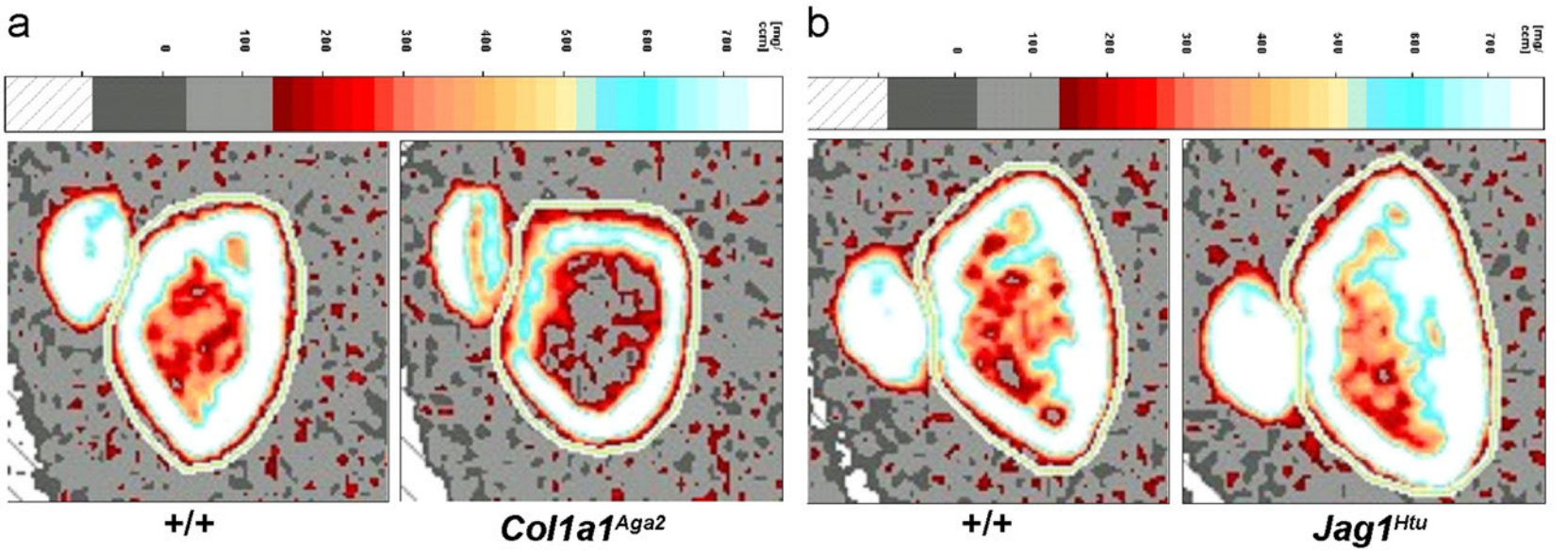
Figure 5

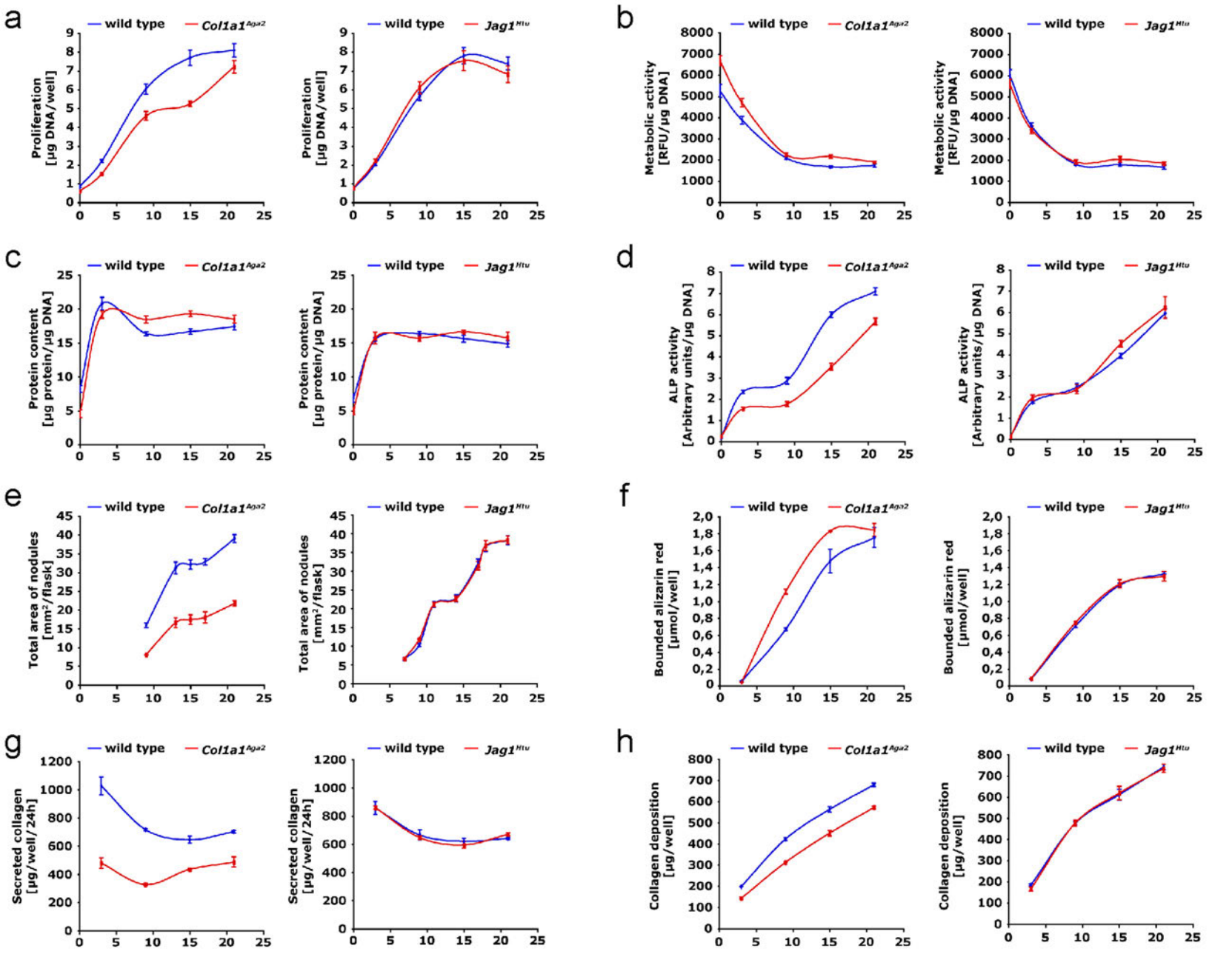
Gene expression analysis. **a** The results of the gene expression analysis are tabularized for *Col1a1*^{Aga2} and *Jag1*^{Htu}. The level of expression difference

between wild-type mice and mutants are indicated by colored boxes. Upregulation is indicated by yellow and red boxes for > 25% and > 50% expression difference, respectively. Downregulation is represented by light green and green for > 25% and > 50% expression differences, respectively. **b,c** The expression of *Col1a1* and *Alpl* are exemplarily shown by diagrams.


3-6 days
5 days
21 days
5 days
Genotyping
Pre-culture
3 week culture period
Measurement days (T0-T21)
Assay analysis
Birth
Cell preparation
Stimulation (T0)
T3
T9
T15
T21


a
b
c
d
e
250000 cells
500000 cells
Number of nodules [per flask]
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
25
Time after stimulation
f
250000 cells
500000 cells
Total area of noduled [mm²/flask]
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Time after stimulation


a
0
100
200
300
400
500
600
700
[mg/ ccm]
+/+
Col1a1Aga2
b
0
100
200
300
400
500
600
700
[mg/ ccm]
+/+
Jag1Htu

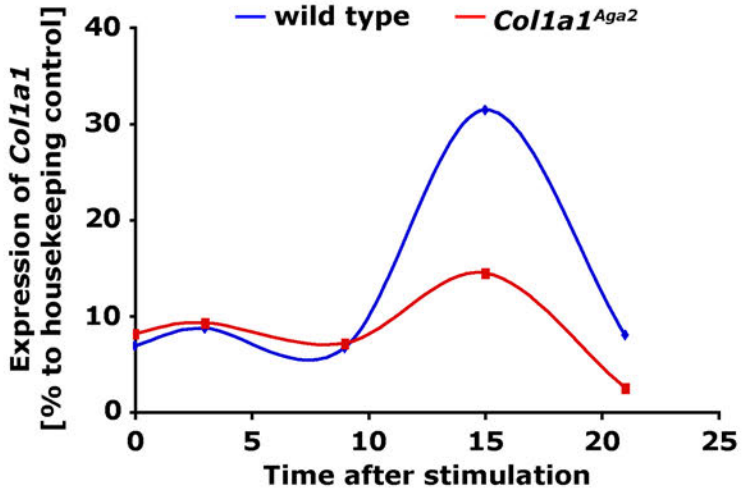

a
wild type
Col1a1Aga2
Jag1Htu
Proliferation [µg DNA/well]
b
Metabolic activity [RFU/µg DNA]
c
Protein content [µg protein/µg DNA]
d
ALP activity [Arbitrary units/µg DNA]
e
Total area of nodules [mm²/flask]
f
Bounded alizarin red [µmol/well]
g
Secreted collagen [µg/well/24h]
h
Collagen deposition [µg/well]

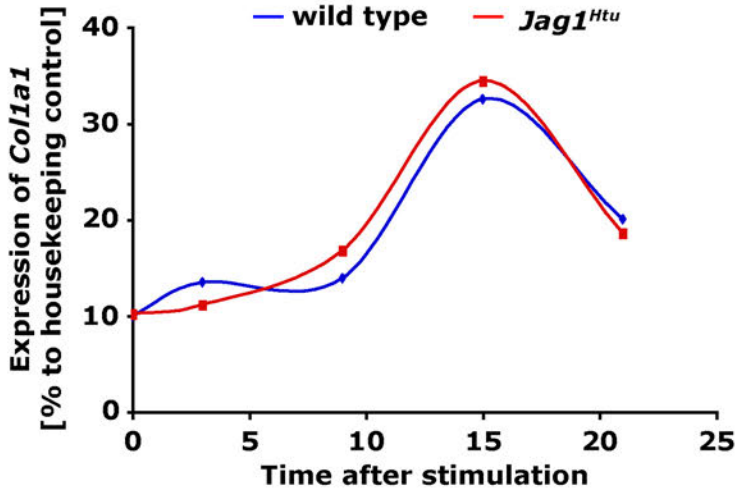
a

Gene	Pheno-type	Time point after stimulation (Expression in % to housekeeping control *Actin beta* + *Pgk1*)				
		0	3	9	15	21
Alpl	WT	6.8	8.6	6.6	31.4	8.0
	Aga2	8.1	9.2	7.1	14.4	2.4
Bglap1	WT	1.3	7.4	15.4	32.5	16.3
	Aga2	1.4	5.3	16.2	15.8	4.5
Col1a1	WT	1302.6	1633.4	980.3	1182.8	507.7
	Aga2	1221.7	1370.0	650.4	676.3	271.4
Cst3	WT	51.0	45.3	45.2	91.0	75.4
	Aga2	45.1	47.9	49.5	91.9	68.4
Pdpn	WT	4.8	3.4	10.4	5.4	4.3
	Aga2	8.2	5.7	8.8	5.7	5.6
Fos	WT	0.8	0.5	0.5	0.5	0.4
	Aga2	0.7	0.6	0.5	0.6	0.5
Fosl2	WT	5.6	3.4	3.3	2.4	2.1
	Aga2	4.6	3.0	2.5	2.2	2.5
Ibsp	WT	0.6	30.7	15.2	103.7	39.6
	Aga2	0.6	17.4	12.6	46.0	9.0
Mmp13	WT	37.4	42.7	145.1	74.7	64.4
	Aga2	79.1	62.0	69.7	54.3	46.5
Sp7	WT	5.2	2.1	4.6	5.9	1.5
	Aga2	4.8	2.5	2.4	2.5	0.4
Runx	WT	2.3	0.9	1.9	1.5	0.9
	Aga2	1.9	1.2	1.1	1.0	0.6
Spp1	WT	64.4	66.0	533.1	368.1	283.9
	Aga2	98.8	61.2	302.1	218.1	230.7
Twist1	WT	3.5	2.1	3.6	3.9	2.5
	Aga2	4.2	2.8	2.8	3.6	2.2

Gene	Pheno-type	Time point after stimulation (Expression in % to housekeeping control *Actin beta* + *Pgk1*)				
		0	3	9	15	21
Alpl	WT	9.9	13.5	13.9	32.5	20.0
	Htu	10.2	11.1	16.8	34.4	18.6
Bglap1	WT	0.7	18.5	22.1	31.0	29.9
	Htu	1.0	27.2	37.3	43.2	36.3
Col1a1	WT	1119.0	1717.4	766.9	1139.8	830.1
	Htu	898.7	1617.9	928.7	1093.9	594.9
Cst3	WT	39.6	40.8	36.1	60.0	73.5
	Htu	40.7	37.2	35.8	56.6	76.9
Pdpn	WT	3.8	3.9	4.6	5.0	4.6
	Htu	3.9	3.0	4.5	4.6	3.7
Fos	WT	1.7	1.5	1.0	0.3	0.4
	Htu	1.5	1.6	1.4	0.3	0.3
Fosl2	WT	6.5	2.6	2.4	1.8	1.9
	Htu	5.4	2.9	3.3	2.1	1.6
Ibsp	WT	0.7	78.1	56.7	191.7	99.9
	Htu	1.6	92.3	102.2	218.7	111.6
Mmp13	WT	51.2	58.2	65.4	40.9	67.3
	Htu	54.2	43.6	79.2	67.4	39.8
Sp7	WT	4.7	4.4	2.4	2.5	2.1
	Htu	4.3	3.8	2.9	2.7	1.9
Runx	WT	1.5	0.9	0.7	1.0	0.9
	Htu	1.3	0.8	0.8	1.0	0.9
Spp1	WT	56.7	71.5	334.8	340.6	318.0
	Htu	56.8	48.9	374.9	398.4	218.3
Twist1	WT	1.5	1.7	1.1	1.3	1.9
	Htu	1.8	1.1	1.2	1.5	1.5

b





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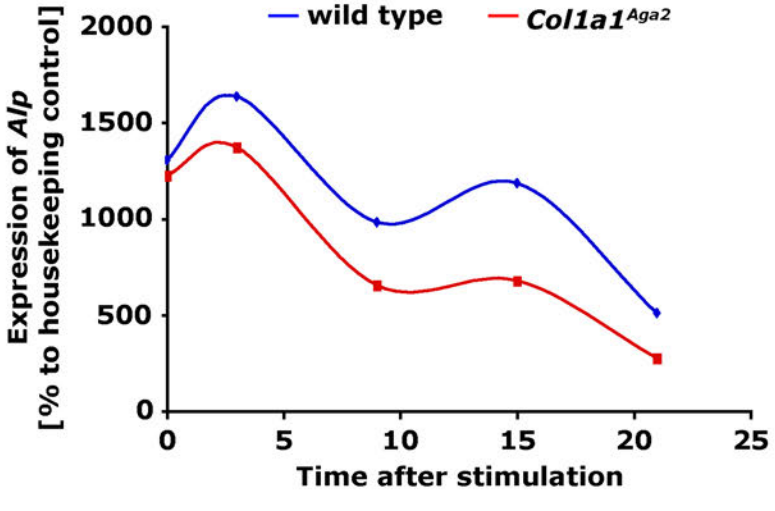
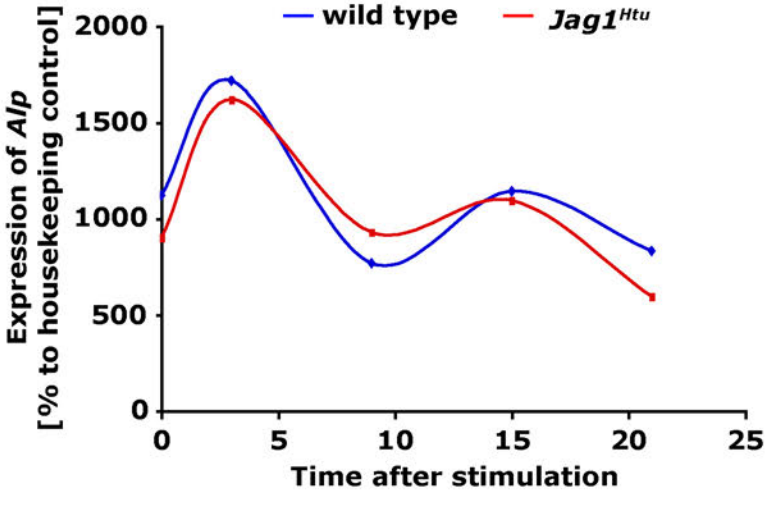




SOP – In vitro analysis of osteoblasts

1. Cell preparation and cultivation

1.1. Material

- EDTA
- 1x PBS (Lonza / BE17-516F)
- Collagenase IV (Sigma-Aldrich / C5138-500MG / charge of importance)
- α-MEM (Lonza / BE12-169F)
- FCS (Gibco / 10500-064 / charge of importance)
- Glutamine (Lonza / BE17-605E)
- Penicillin / streptomycin (Lonza / DE17-602E)
- Ascorbic acid (Sigma-Aldrich / A4544-25G)
- β-glycerol phosphate (AppliChem / A2253,0100)
- Ethanol 70%
- Trypan blue 0.4%

- 15 ml / 50 ml tubes
- Petri dishes
- 0.22 µm syringe filter (Millipore / SLGP033RS)
- 40 µm cell strainer (BD / 3523409)
- 0.2 µm bottle-top-filter (NeoLab / 431097)
- 24-well Lumox™ cell culture plates (Greiner bio one / 96110024)
- 24-well Primaria™ cell culture plates (BD / 353847)
- 6-well Primaria™ cell culture plates (BD / 353846)
- T12.5 cm^2 cell culture flasks (BD / 353107)
- Dissecting set
- Neubauer cell counting chamber
- 37°C water bath
- Laminar flow
- Cell culture incubator

- 0.1% Collagenase IV in 1x PBS (freshly made / filtration through 0.22 µm syringe filter)
- 4 mM EDTA in 1x PBS (ph7) (superposable)
- Culture medium A:
    - α-MEM supplemented with 10% FCS / 2mM glutamine / 100 U/ml penicillin / 100 µg/ml streptomycin
- Culture medium B:
    - α-MEM supplemented with 10% FCS / 2mM glutamine / 100 U/ml penicillin / 100 µg/ml streptomycin / 10 mM β-glycerol phosphate / 50 µg/ml ascorbic acid

1.2. Method

- Preparation of mutant and wild-type control mice is performed in parallel but in two separate groups (mut / wt)
- After decapitation of mice the skull is briefly immerged in 70% ethanol and afterwards kept on ice in 1x PBS
- The following steps are performed under a laminar flow
- Calvariae are dissected by cutting the skin from posterior (foramen magnum) to anterior (nose), the skin is removed and the calvaria is carefully taken off from the brain
- Calvariae are placed in a petri dish with 1x PBS and superfluous contaminating tissues are removed
- For each group (mut / wt): 4-5 calvariae are placed in a 15 ml tube - it is advisable to split the total number of calvariae from each group in 4 x 15 ml tubes (correspond to 4 tubes with 5 calvaria each if preparing 20 calvaria per group)
- Each tube is incubated twice with 4-5 ml (1 ml per calvaria) 4 mM EDTA in 1x PBS for 7 min at 37°C (water bath), the supernatant is discarded
- Each tube is washed three times with 4-5 ml (1 ml per calvaria) 1x PBS
- Predigestion: each tube is incubated once with 2 ml 0.1% Collagenase IV for 7 min at 37°C (water bath) and the supernatant is discarded
- Digestion step 1: each tube is incubated with 5 ml 0.1% Collagenase IV for 30 min at 37°C (water bath) under occasionally agitation, the supernatant from the digestion of each group (4 x 15 ml tubes a 5 ml) is filtered through a 44 µm cell strainer in one 50 ml tube and 20 ml culture medium A is added (yield in 40 ml cell suspension per group) - attention should be paid to retain the undigested remains of the calvariae in the 15 ml tubes for the second digestion step
- Digestion step 2: equal to digestion 1
- During the second digestion both 50 ml tubes with the cell suspension of the first digestion (1 x wt / 1x mut) are kept at 37°C in the incubator
- The resulting 4 x 50 ml tubes of both digestion steps (2 x wt / 2 x mut) are centrifuged for 10 min / 1200 rpm / RT
- For each group: the supernatant is discarded and the cell pellets of both 50 ml tubes are resuspended and put together in 10 ml culture medium A, the quantity of cells is estimated using the Neubauer counting chamber and cells are plated according to table 1 with $5x10^4$ cells / well in 24-well plates, $2x10^5$ cells / well in 6-well plates and $2.5x10^5$ cells / flask in T12.5 cm^2 cell culture flasks
- The medium is changed 24 h after plating using culture medium A and thereafter, the medium is regularly changed twice a week (monday / thursday or tuesday / friday), starting with the first stimulation at T0 using culture medium B

- An overview about the different assays including a schedule for sample taking is depicted in table 2

	Assay	Type of culture vessel	Number of culture vessel*	Distribution of cells in vessel
A1	Proliferation Metabolic activity Protein content ALP activity	24-well Lumox™	5	6 wells wt 6 wells mut
A2	Collagen secretion Collagen deposition	6-well Primaria™	4	3 wells wt 3 wells mut
A3	Matrix mineralization	6-well Primaria™	4	3 wells wt 3 wells mut
A4	Nodule quantification	T12.5cm^2	8$^{\#}$	4 flasks wt 4 flasks mut
A5	Gene expression	24-well Primaria™	5	6 wells wt 6 wells mut

Table 1. Overview about culture ware for the cell culture system. * number of culture vessels equates number of measurement days for sample taking. # number of flasks does not correspond to measurement days but equates the number of biological replicates used for nodule quantification (4 x wt / 4 x mut), since each flask is used throughout the whole culture period for every single measurement day.

	Assay	Measurement days				
		T0	T3	T9	T15	T21
A1	Proliferation Metabolic activity Protein content ALP activity	X X X X	X X X X	X X X X	X X X X	X X X X
A2	Collagen secretion Collagen deposition		X X	X X	X X	X X
A3	Matrix mineralization		X	X	X	X
A4	Nodule quantification	different measurement days*				
A5	Gene expression	X	X	X	X	X

Table 2. Assays and measurement scheme of the cell culture system. *starting with T3, this assay is performed 3x a week (Monday / Wednesday / Friday) until T21. The left column (A1-A5) provides an overview if cultivated cells are applied for one or for multiple assays (e.g. in A1 the same cells are used for 4 different assays while in A3 the assay is performed with a single batch of cells).

2. Proliferation / Metabolic activity / Protein content / ALP activity (A1)

2.1. Material

- CellQuanti-Blue™ (BioAssay Systems; Biotrend Chemikalien GmbH / CQBL-05K)
- CellyticM (Sigma-Aldrich / C2978-50ML)
- Complete Mini Protease Inhibitor (Roche / 11836153001)
- Quant-iT™ dsDNA Assay Kit Broad Range (Invitrogen / Q33130)
- BCA-Protein Assay Kit (Thermo Scientific / 23225)
- 4-Nitrophenylphosphate (PNPP) (Sigma-Aldrich / N4645-1G)
- Proteinase K (20 mg/ml)
- EDTA
- Glycine
- $MgCl_2$
- NaCl
- NaOH
- SDS
- Tris
- $ZnCl_2$

- 96-well plate black (Nunc / 237107)
- 96-well plate transparent (Nunc / 260860)
- Bench top centrifuge with refrigeration to 4°C
- Fluorescence micro plate reader (Tecan Safire^2)
- Spectrophotometer for 96-well plates (Molecular Devices / SpectraMax 190) including software (SOFTmaxPRO 3.1.2)

- SDS-buffer (for 200ml / superposable):
    - 2% SDS
    - 10 mM Tris
    - 5 mM EDTA
    - 200 mM NaCl
- ALP-buffer (for 100ml / superposable):
    - 0,1 M glycine (pH 9,6)
    - 1 mM $MgCl_2$
    - 1 mM $ZnCl_2$
- PNPP-buffer (for 500ml / superposable):
    - 3,75 g glycine
    - 1 mM $MgCl_2$
    - 1 mM $ZnCl_2$
    - dissolve in in 400 mL H_2O
    - adjust pH to 10,4 using NaOH
    - fill up to 500 ml with H_2O
- PNPP in PNPP-buffer (freshly made):
    - 1 ml PNPP-buffer + 20 mg PNPP

2.2. Measurement day

- 24-well lumox™ cell culture plate
- The samples (n wells) of both biological groups are prepared separately (technical replicates)
    - 6 x wt
    - 6 x mut

- CellQuanti-Blue™ reagent is diluted 1:10 with culture medium A (CQBM-solution)
- Aspiration of old media from the wells
- 1 ml CQBM solution is added per well and incubated for 1 h at 37°C (the lower row of wells without cells is used as blank by adding 1 ml CQBM solution per well)
- Fluorescence spectroscopy with the cell culture plate (Tecan – Safire^2)
    - extinction: 530 nm
    - emission: 590 nm
    - band width: 10 nm
    - z-value: 11200 µm
    - gain: 65 nm (manual)
    - range of temperature: 36.5 – 37.5°C
    - measurement is saved → Metabolic activity

- Preparation of cell lysis reagent: 10 ml CellyticM + 1 tablet Complete Mini Protease Inhibitor (CMCM solution)
- CQBM solution is aspirated and cells are washed once with 1x PBS
- 500 µl CMCM solution is added per well and incubated for 15 min at 4°C under continuous shaking (orbital shaker)
- 4°C room: supernatant of each well is transfered under multiple up and down pipetting (complete lysis of cells) in 1.5 ml Eppendorf tubes and centrifuged for 10 min / 4°C / 12000 rpm
- 350 µl supernatant is carefully transfered in new 1.5 ml tube and stored at -20°C (tube 1) → Protein content / ALP activity
- Pellet (contains nuclei) including remaining 150 µl supernatant is stored at -20°C (tube 2) → Proliferation (DNA-contant)

2.3. Assay analysis

→ Metabolic activity

- The metabolic activity of the cells is directly obtained and saved as fluorescent value per sample (well) at every measurement day [fluorescence/well]
- A standard curve is not applied, metabolic activity is described as raw fluorescence value (RFU)

→ Protein content

- An existing standard curve is applied for the analysis
    - the standard curve is generated according to the protocol of the BCA Protein Assay Kit with µg/ml protein (x-axis) against OD (y-axis), saved and reused for every analysis
- For each group (wt = 30 samples / mut = 30 samples) one 96-well plate (transparent) is used, samples can be measured in duplicate
- Two plates are needed, each plate is prepared according to the following scheme
    - 30 samples in duplicate (60 wells)
    - blank (CMCM solution) in duplicate (2 wells)

- Tube 1 (350 µl supernatant) is thawed at 4°C
- Analysis of protein is performed using the BCA Protein Assay Kit in the 96-well plates
    - working solution (WS) is prepared: 50 units BCA reagent A + 1 unit BCA reagent B
    - samples: 25 µl of lysate (tube 1) per well
    - blank: 25 µl CMCM solution per well
    - 200 µl WS is added to each well and both plates are incubated for 30 min at 37°C
    - photometric absorption reading (photometer Spectramax)
        - endpoint reading
        - wave length: 562 nm

- The protein content of each sample is calculated according to the following scheme
    - both plates (wt / mut) are analysed separately
    - standard curve with [µg/ml] of protein
    - automatically via SOFT maxPRO: blank value is subtracted from the absorption values of the samples, thereafter the protein content of each sample is calculated on the basis of the standard curve in [µg/well]
    - please note: the applied volume of 500 µl CMCM solution per well (for cell lysis at the measurement days) has to be taken into account for the calculation

→ ALP activity

- A standard curve is not applied, ALP activity is described as absorption value (arbitrary units)
- For each group (wt = 30 samples / mut = 30 samples) one 96-well plate (transparent) is used, samples can be measured in duplicate
- Two plates are needed, each plate is prepared according to the following scheme
    - 30 samples in duplicate (60 wells)
    - blank (CMCM solution) in duplicate (2 wells)

- Tube 1 (350 µl supernatant) is thawed at 4°C
- Analysis of ALP activity is performed in the 96-well plates according to the following procedure
    - working solution (WS) is prepared: 9 units ALP-buffer + 1 unit PNPP in PNPP-buffer
    - samples T0: 100 µl lysat (tube 1) per well (undiluted)
    - samples T3-T21: 5 µl lysat (tube 1) + 95 µl H_2O per well (1:20)
    - blank: 5 µl CMCM solution + 95 µl H_2O per well (1:20)
    - 100 µl WS is added to each well and both plates are incubated for 30 min at 37°C
    - 50 µl 1 M NaOH is added to each well
    - photometric absorption reading (photometer Spectramax)
        - endpoint reading
        - wave lenght: 405 nm

- The ALP activity of each sample is calculated according to the following scheme
    - both plates (wt / mut) are analysed separately
    - without standard curve
    - automatically via SOFT maxPRO: blank value is subtracted from the absorption values of the samples, thereafter the ALP-activity of each sample is described as absolute absorption value in [absorption/well]
    - please note: from the 500 µl CMCM solution per well (for cell lysis at the measurement days) only 100 µl were used for the analysis either undiluted (T0) or 1:20 diluted (T3-T21)

→ Proliferation (DNA content)

- A standard curve is used for the analysis and prepared for each measurement (each plate)
    - the standard curve is described as µg DNA (x-axis) against fluorescence (y-axis)
- Both groups (wt = 30 samples / mut = 30 samples) are analysed on one 96-well plate (black), the samples can not be measured in duplicate
- To still analyze each sample in duplicate, two plates are used and identically prepared according to the following scheme
    - 60 samples (60 wells)
    - 8 standard concentrations (0 – 1 µg) in duplicate (16 wells)
    - blank (1:1 mixture of CMCM solution and SDS-buffer with Proteinase K) in triplicate (3 wells)

- Tube 2 (pellet including 150 µl supernatant) is thawed at 4°C
- 150 µl SDS-buffer and 1.5 µl Proteinase K is added to each well (yield in 300 µl lysat), vortexed, spinned down and incubated for 3 h at 56°C using a thermo shaker (750 rpm)
- Analysis of DNA content is performed using the Quant-iT™ DNA Assay Kit Broad Range in the 96-well plates
    - working solution (WS) is prepared: Quant-iT™ reagent is diluted 1:200 with Quant-iT™ buffer
    - samples: 10µl lysat (tube 2) + 10 µl H_2O per well
    - standard: 10µl blank (1:1 mixture of CMCM solution and SDS-buffer with Proteinase K) + 10 µl of each DNA standard concentration from the kit per well
    - blank: 10 µl blank (1:1 mixture of CMCM solution and SDS-buffer with Proteinase K) + 10 µl H_2O per well
    - 200 µl WS is added to each well and both plates are incubated for 5 min at RT
    - fluorescence spectroscopy (Tecan – Safire^2)
        - extinction: 490nm
        - emission: 527nm
        - band width: 10 nm
        - gain: optimal

- DNA content of each sample is calculated according to the following scheme
    - both plates (wt / mut) are analysed separately
    - standard curve with [µg] of DNA (based on the fluorescence values of the standards of each plate)
    - blank value is subtracted from the fluorescence values of the samples, thereafter the DNA content of each sample is calculated on the basis of the standard curve in [µg/well]
    - please note: from the 300 µl overall lysate per sample (150 µl pellet + 150 µl SDS-buffer) only 10 µl were used for the analysis
    - the mean value from both measurements (plate 1 / plate 2) result in the DNA content of each sample

2.4. Results and presentation

→ Proliferation (DNA content)

- For each sample (well) the DNA content is determined in [µg DNA/well]
- For each measurement day the mean value of each group (wt / mut) is calculated by averaging the 6 single values of each group (6 x wt / 6 x mut)
- For graphic presentation of the proliferation during the culture period, a combined diagram for both groups is drawn with the obtained values on the y-axis [µg DNA/well] against the measurement days on the x-axis

→ Metabolic activity

- For each sample (well) the metabolic activity is determined in [fluorescence (RFU)/well]
- To normalize the metabolic activity to the cell number, the value is divided by the DNA content of the sample to obtain the metabolic activity per DNA amount in [fluorescence (RFU)/µg DNA] for each sample
- For each measurement day the mean value of each group (wt / mut) is calculated by averaging the 6 single values of each group (6 x wt / 6 x mut)
- For graphic presentation of the metabolic activity during the culture period, a combined diagram for both groups is drawn with the obtained values on the y-axis [fluorescence (RFU)/µg DNA] against the measurement days on the x-axis

→ Protein content

- For each sample (well) the protein content is determined in [µg protein/well]
- To normalize the protein content to the cell number, the value is divided by the DNA content of the sample to obtain the protein content per DNA amount in [µg protein/µg DNA] for each sample
- For each measurement day the mean value of each group (wt / mut) is calculated by averaging the 6 single values of each group (6 x wt / 6 x mut)
- For graphic presentation of the protein content during the culture period, a combined diagram for both groups is drawn with the obtained values on the y-axis [µg protein/µg DNA] against the measurement days on the x-axis

→ ALP activity

- For each sample (well) the ALP activity is determined in [absorption (arbitrary units)/well]
- To normalize the ALP activity to the cell number, the value is divided by the DNA content of the sample to obtain the ALP activity per DNA amount in [absorption (arbitrary units)/µg DNA] for each sample
- For each measurement day the mean value of each group (wt / mut) is calculated by averaging the 6 single values of each group (6 x wt / 6 x mut)
- For graphic presentation of the ALP activity during the culture period, a combined diagram for both groups is drawn with the obtained values on the y-axis [absorption (arbitrary units)/µg DNA] against the measurement days on the x-axis

3. Collagen secretion / Collagen deposition (A2)

3.1. Material

- Bouin's solution (Sigma-Aldrich / HT10132-1L)
- Sircol™ Dye reagent (from Sircol Collagen Assay Kit) (Biocolor; Tebu-bio / S1005)
- Sirius Red FB3 (Fluka; Sigma-Aldrich / 43665)
- Saturated picric acid solution (Fluka; Sigma-Aldrich / 80456)
- Picric acid (Sigma-Aldrich / 239801-50G)
- Collagen solution
- HCl / NaOH

- 96-well plate transparent (Nunc / 260860)
- Spectrophotometer for 96-well plates (Molecular Devices / SpectraMax 190) including software (SOFTmaxPRO 3.1.2)

- Culture medium C:
  - α-MEM supplemented with 5% FCS / 2 mM glutamine / 100 U/ml penicillin / 100 µg/ml streptomycin / 10 mM β-glycerol phosphate / 50 µg/ml ascorbic acid
- Sirius Red solution:
  - 0.25 g Sirius Red FB3
  - 250 ml saturated picric acid solution
  - 1 ml picric acid (to ensure saturation of the solution)

- Please note: the medium has to be changed in the cells to be analyzed 24 h before the measurement day
  - 6-well Primaria™ cell culture plate
  - aspiration of old media from the wells
  - 2 ml culture medium C is added per well and incubated for 24 h at 37°C

3.2. Measurement day

- 6-well Primaria™ cell culture plate with culture medium C for 24 h
- The samples (n wells) of both biological groups are prepared separately (technical replicates)
  - 3 x wt
  - 3 x mut

- 1ml of the cell culture supernatant (culture medium C) is transferred in a 1.5 ml tube and stored at -20°C → Collagen secretion
- Aspiration of the remaining media from the wells
- Cells are washed once with 1x PBS
- 2ml Bouin's solution is added to each well and incubated for 60 min at RT
- Bouin's solution is aspirated and cells are washed twice with H_2O for 5 min at RT each
- 5 ml of H_2O and 200 µl Penicillin / Streptomycin solution is added to each well, the plates are sealed with parafilm and stored in a closed box (e.g. styrofoam-box) at RT → Collagen deposition

3.3. Assay analysis

→ Collagen secretion

- An existing standard curve is applied for the analysis
  - the standard curve is generated according to the protocol explained below (3.4.) with µg/ml collagen (x-axis) against OD (y-axis), saved and reused for every analysis
- The Sircol™ Dye reagent (from Sircol Collagen Assay Kit) is used for the analysis
- Both groups (wt = 12 samples / mut = 12 samples) are analyzed on one 96-well plate (transparent), the samples can be measured in duplicate, the plate is prepared according to the following scheme
  - 24 samples in duplicate (48 wells)
  - blank (culture medium C) in duplicate (2 wells)

- Supernatant is thawed at 4°C
- 200 µl of the supernatant of each sample is transferred to a new 1.5 ml tube
- Preparation of blank: 200 µl of fresh culture medium C is transferred to a new 1.5 ml tube
- 1 ml Sircol™ Dye reagent is added, tubes are briefly vortexed, spinned down and incubated for 30 min at RT under continuing shaking (750 rpm on shaker)
- Tubes are centrifuged for 10 min / RT / 13000 rpm
- Supernatant is discarded and tubes are centrifuged for another 5 min / RT / 13000 rpm
- Remaining supernatant is carefully removed via pipette tip (contact of pipette tip with pellet has to be avoided)
- 1 ml 0.5 M NaOH is added, the tubes are briefly vortexed, spinned down and incubated for 10 min at RT under continuing shaking (750 rpm on shaker) → pellet has to be completely dissolved (tubes have to be vortexed again if necessary)
- The Sircol™ Dye reagent that was bounded to the collagen of the cell culture supernatant (pellet) is unhinged by the NaOH and the concentration of the dye in each sample is determined by photometric absorption reading in a 96-well plate according to the following scheme:
  - samples and blank: 40 µl NaOH-Sircol™ Dye solution and 160 µl H_2O is added per well (1:5)
  - photometric absorption reading (photometer Spectramax)
    - endpoint reading
    - wave length: 550 nm

- The absorption values correlate with the amount of bounded Sircol™ Dye reagent, that is proportional to the concentration of collagen in the respective sample (200 µl cell culture supernatant); therefore, the amount of collagen in each sample can be calculated by the absorption values according to the following scheme
  - standard curve with [µg/ml] collagen
  - automatically via SOFT maxPRO: blank value is subtracted from the absorption values of the samples, thereafter the amount of collagen in each sample is calculated on the basis of the standard curve in [µg/well]

- please note: given that 1 ml NaOH was applied for unhinge the Sircol™ Dye reagent from the pellet and that the standard curve is depicted in µg/ml, the amount of collagen can directly be obtained from the standard curve, but it has to be taken into account, that only 200 µl from the 2 ml cell culture supernatant of each well were used for the analysis and that the NaOH-Sircol™ Dye solution was 1:5 diluted for the absorption reading

→ Collagen deposition

- An existing standard curve is applied for the analysis
  - the standard curve is generated according to the protocol explained below (3.4.) with µg/ml collagen (x-axis) against OD (y-axis), saved and reused for every analysis
- The Sirus Red solution (self-made) is used for the analysis
- Both groups (wt = 12 samples / mut = 12 samples) are analyzed on one 96-well plate (transparent), the samples can be measured in duplicate, the plate is prepared according to the following scheme
  - 24 samples in duplicate (48 wells)
  - blank (NaOH) in duplicate (2 wells)

- Aspiration of the water in the wells
- 2 ml Sirius Red solution is added to each well and incubated for 1 h at RT
- Sirius Red is aspirated and cells are washed twice with H_2O for 5 min at RT
- Cells are washed once with 0.01 M HCl for 2 min at RT
- 1 ml 0.5 M NaOH is added per well and incubated for 60 min at RT
- The Sirius Red solution that was bounded to the collagen of the cell matrix (well) is unhinged by the NaOH and the concentration of the dye in each sample is determined by photometric absorption reading in a 96-well plate according to the following scheme:
  - samples: 20 µl NaOH-Sirius Red solution and 180 µl H_2O are added per well (1:10)
  - blank: 20 µl fresh NaOH solution and 180 µl H_2O are added per well (1:10)
  - photometric absorption reading (photometer Spectramax)
    - endpoint reading
    - wave length: 550 nm

- The absorption values correlate with the amount of bounded Sirius Red solution, that is proportional to the concentration of collagen in the respective sample (well), therefore, the amount of collagen in each sample can be calculated by the absorption values according to the following scheme
  - standard curve with [µg/ml] collagen
  - automatically via SOFT maxPRO: blank value is subtracted from the absorption values of the samples, thereafter the amount of collagen in each sample is calculated on the basis of the standard curve [µg/well]
  - please note: given that 1 ml NaOH was applied for unhinge the Sirius Red solution from the well and that the standard curve is depicted in µg/ml, the amount of collagen can directly be obtained from the standard curve, but it has to be taken into account, that the NaOH-Sirius Red solution was 1:10 diluted for the absorption reading

3.4. Generation of the standard curve for Collagen secretion / deposition

- Material: 96-well plate (transparent) / collagen solution [1mg/ml] / 0.1 M NaOH / Sircol™ Dye reagent (from Sircol Collagen Assay Kit)

- Using the collagen solution [1mg/ml] 6 standard samples are prepared in 1.5 ml tubes with an amount of 0 / 20 / 40 / 60 / 80 / 100 µg collagen
- 1 ml Sircol™ Dye reagent is added to each standard sample, the tubes are briefly vortexed, spinned down and incubated for 30 min at RT under continuing shaking (750 rpm on shaker)
- Centrifugation for 10 min / RT / 13000 rpm
- Supernatant is discarded and the tubes are centrifuged for another 5 min / RT / 13000 rpm
- Remaining supernatant is carefully removed via pipette tip (contact of pipette tip with pellet has to be avoided)
- 1 ml 0.5 M NaOH is added, the tubes are briefly vortexed, spinned down and incubated for 10 min at RT under continuing shaking (750 rpm on shaker) → pellet has to be completely dissolved (tubes have to be vortexed again if necessary)
- The Sircol™ Dye reagent that was bounded to the collagen in the standard samples (pellet) is unhinged by the NaOH and the concentration of the dye in each standard sample is determined by photometric absorption reading in a 96-well plate according to the following scheme:
    - standards (in duplicate): 200 µl NaOH-Sircol™ Dye solution are added per well
    - blank (in duplicate): 200 µl fresh NaOH is added per well
    - photometric absorption reading (photometer Spectramax)
        - endpoint reading
        - wave length: 550 nm

- The absorption values correlate with the amount of bounded Sircol™ Dye reagent, that is proportional to the concentration of collagen in the respective standard samples (0 / 20 / 40 / 60 / 80 / 100 µg), therefore, a standard curve can be generated with µg/ml collagen (x-axis) against OD (y-axis), saved and reused for every analysis

3.5. Results and presentation

→ Collagen secretion

- For each sample (well) the amount of secreted collagen is determined in [µg collagen/well]
- The value correspond to the overall amount of secreted collagen from the cells of one sample (well) inbetween 24h of incubation [µg collagen/well/24h]
- For each measurement day the mean value of each group (wt / mut) is calculated by averaging the 3 single values of each group (3 x wt / 3 x mut)
- For graphic presentation of the collagen secretion during the culture period a combined diagram for both groups is drawn with the obtained values on the y-axis [µg collagen/well/24h] against the measurement days on the x-axis

→ Collagen deposition

- For each sample (well) the amount of deposited collagen is determined in [µg collagen/well]
- For each measurement day the mean value of each group (wt / mut) is calculated by averaging the 3 single values of each group (3 x wt / 3 x mut)
- For graphic presentation of the collagen deposition during the culture period a combined diagram for both groups is drawn with the obtained values on the y-axis [µg collagen/well] against the measurement days on the x-axis

4. Matrix mineralization (A3)

4.1. Material

- 4% paraformaldehyde
- Alizarin Red S (Sigma-Aldrich / A5533-25G)
- Cetylpyridinium chloride (Sigma-Aldrich / C5460-100G)

- 96-well plate transparent (Nunc / 260860)
- Spectrophotometer for 96-well plates (Molecular Devices / SpectraMax 190) including software (SOFTmaxPRO 3.1.2)

- 5% Alizarin Red S stock solution in H_2O (ph 4.0 / filter sterile 0.22 µm / superposable)
- 0.5% Alizarin Red S working solution in H_2O (freshly made)
- 10% cetylpyridinium chloride solution in H_2O (freshly made)

4.2. Measurement day

- 6-well Primaria™ cell culture plate
- The samples (n wells) of both biological groups are prepared separately (technical replicates)
    - 3 x wt
    - 3 x mut

- Aspiration of old media from the wells
- Cells are washed once with 1x PBS
- 2 ml 4% PFA is added per well and incubated for 15 min at 4°C
- PFA is aspirated and cells are washed twice for 5 min at RT with H_2O
- Plates are air-dried and afterwards stored in a closed box (e.g. styrofoam-box) at RT

4.3. Assay analysis

- An existing standard curve is applied for the analysis
    - the standard curve is generated according to the protocol explained below (4.4.) with µM Alizarin (x-axis) against OD (y-axis), saved and reused for every analysis

- Both groups (wt = 12 samples / mut = 12 samples) are analyzed on one 96-well plate (transparent), the samples can be measured in duplicate, the plate is prepared according to the following scheme
    - 24 samples in duplicate (48 wells)
    - blank (10% cetylpyridinium chloride) in duplicate (2 wells)

- 2 ml 0.5% Alizarin Red S is added per well and incubated for 60 min at RT
- Alizarin is aspirated and cells are washed 4 times with H_2O for 5 min at RT each
- 1 ml 10% cetylpyridinium chloride is added per well and incubated for 60 min at RT

- Bounded Alizarin is unhinged by the cetylpyridinium chloride and the concentration of the dye in each sample is determined by photometric absorption reading in a 96-well plate according to the following scheme:
    - samples T3: 200 µl of the cetylpyridinium chloride-Alizarin solution is added per well (undiluted)
    - samples T9-T21: 40 µl of the cetylpyridinium chloride-Alizarin solution and 160 µl H_2O are added per well (1:5)
    - blank: 40 µl fresh cetylpyridinium chloride and 160 µl H_2O are added per well (1:5)
    - photometric absorption reading (photometer Spectramax)
        - endpoint Reading
        - wave length: 562 nm

- The absorption values correlate with the concentration of Alizarin Red S in the respective sample and therefore, the amount of bounded Alizarin in each well (sample) can be calculated according to the following scheme
    - standard curve with [µM] Alizarin
    - automatically via SOFT maxPRO: blank value is subtracted from the absorption values of the samples, thereafter the amount of bounded Alizarin of each sample is calculated on the basis of the standard curve in [µmol/well]
    - please note: the applied volume of 1ml cetylpyridinium chloride per well and the 1:5 dilution for absorption reading in the samples T9-T21 (T3 undiluted) has to be taken into account for the calculation

4.4. Generation of the standard curve for Matrix mineralization

- Material: 96-well plate (transparent) / 0.5% Alizarin Red S (=14.6 mM) / 10% cetylpyridinium chlorid

- Preparation of 10 standard solutions according to the following scheme

Standard	µl Alizarin Red S	µl cetylpyridinium chloride	Final concentration in µM*
1	0	1000	0
2	0.68	999.32	2
3	3.4	996.6	10
4	6.84	993.16	20
5	17.1	982.9	50
6	34.2	965.8	100
7	68.4	931.6	200
8	136.8	863.2	400
9	205.2	794.8	600
10	273.6	726.4	800

Preparation of a standard curve for the Matrix mineralization assay. * final concentration already including a 1:5 dilution of the standard solutions for the absorption reading

- Concentration of all 10 standards is determined according to the following scheme
    - standards (in duplicate): 40 µl standard solution + 160 µl H_2O is added per well (1:5)
    - blank (in duplicate): 200 µl H_2O is added per well
    - photometric absorption reading (photometer Spectramax)
        - endpoint reading
        - wave length: 562 nm

- The absorption values correlate with the concentration of Alizarin Red S in the respective standards and thus, a standard curve can be generated with µM Alizarin (x-axis) against OD (y-axis), saved and reused for every analysis
- Please note: the final concentration [µM] of the standards depicted in the scheme above already include the 1:5 dilution of the standard solutions for the absorption reading

4.5. Resultats and presentation

- For each sample (well) the amount of bounded Alizarin is determined in [µmol Alizarin/well]
- For each measurement day the mean value of each group (wt / mut) is calculated by averaging the 3 single values of each group (3 x wt / 3 x mut)
- For graphic presentation of the matrix mineralization during the culture period a combined diagram for both groups is drawn with the obtained values on the y-axis [µmol Alizarin/well] against the measurement days on the x-axis

5. Nodule quantification (A4)

5.1. Material

- Stereo microscope equipped with camera and software for image acquisition (Leica MZ16F / DFC320 / Firecam)
- Holding frame for T12.5 cm^2 cell culture flasks (enables identical positioning of the culture flask under the stereo microscope at every measurement day)
- Pixel counting software (Image J 1.38x)

5.2. Measurement day

- T12.5 cm^2 cell culture flasks
- The samples (n cell culture flasks) of both biological groups are used for the whole culture period and are analyzed separately at each measurement day (technical replicates)
    - 4 x wt
    - 4 x mut

- Transmitted light images are taken from all cell culture flasks using a stereo microscope with the following settings (Leica MZ16F / DFC320 / Firecam)
    - Source of light: Leica KL1500LCD / 3200K (5/E)
    - Bright field
    - Objective: 0.63x
    - Magnification: 1.0x
    - Exposure: 10msec
    - Gain: 1.0x
    - Live Video: Progressive VGA
    - Color: Auto
    - Levels: Auto
    - Output: Full Frame HQ 2088 / JPEG
    - to arrange and arrest the flasks in the same position on the microscope stage at each measurement day, they are placed in a special custom-built holding frame for T12.5 cm^2 cell culture flasks
    - the raw images are stored until the Assay analysis

5.3. Assay analysis

- The JPEG images are analyzed using the pixel counting software ImageJ (Version 1.38x) according to the following procedure (all images from both groups are analyzed in parallel in terms of a stack analysis):
    - File → Import → Image Sequence…
    - Process → Substract Background… → Rolling Ball Radius: 50 / Light Background √ → Process all slices yes √
    - Process → Binary → Make Binary → Calculated Threshold for Each Image √
    - Analyze → Set Scale → Distance in Pixel: 97 / Unit of Length: mm / Global √
    - Analyze → Analyze Particle → Display Results √ / Summarize √ → Process all slices √

- for each image the parameter Count / Total Area / Average Size / Area Fraction are obtained and summarized in the Summary Table (according to the parameter, the numerical values correspond to mm or mm^2)
- the results of the Summary Table are transferred into an Excel Table
- the different parameter of an image correspond to the following feature in the respective sample (cell culture flask):
    - Count → number of nodules
    - Total Area → total area of nodules
    - Average Size → average size of a nodule
    - Area Fraction → fraction of nodule area from total area in the flask (%)

5.4. Results and presentation

- For each sample (cell culture flask) the total area of nodules is determined in [mm^2]
- For each measurement day the mean value of each group (wt / mut) is calculated by averaging the 4 single values of each group (4 x wt / 4 x mut)
- For graphic presentation of the nodule development during the culture period a combined diagram for both groups is drawn with the obtained values on the y-axis [mm^2] against the measurement days on the x-axis

6. Gene expression (A5)

6.1. Material

- RNeasy Mini Kit (Qiagen / 74106)
- QIAshredder (Qiagen / 79654)
- β-mercaptoethanol
- SuperScript II Reverse Transcriptase (Invitrogen / 18064-014)
- RNAse OUT Recombinant Ribonuclease Inhibitor (Invitrogen / 10777-019)
- Primer OligodT (15er) (Promega / C110A)
- dNTP's (10mM each)
- *Power* SYBR® Green PCR Master Mix (Applied Biosystems / 4367659)
- Primers according to table 3

Gene	forward primer 5' → 3'	reverse primer 5' → 3'
Alpl	TTGTGCCAGAGAAAGAGAGAGAC	TTGGTGTTATATGTCTTGGAGAGG
Bglap	GACCTCACAGATGCCAAGC	GACTGAGGCTCCAAGGTAGC
Col1a1	CCAAGAAGACATCCCTGAAGTC	TTGGGTCCCTCGACTCCT
Cst3	CGTAAGCAGCTCGTGGCT	CAGAGTGCCTTCCTCATCAGA
Pdpn	CAGATAAGAAAGATGGCTTGCC	CTCTTTAGGGCGAGAACCTTC
Fos	CAACACACAGGACTTTTGCG	TCAGGAGATAGCTGCTCTACTTTG
Fosl2	AGATGAGCAGCTGTCTCCTGA	TCCTCGGTCTCCGCCT
Ibsp	AGGAAGAGGAGACTTCAAACGA	TGCATCTCCAGCCTTCTTG
Mmp13	GCAGTTCCAAAGGCTACAACTT	TCATCGCCTGGACCATAAAG
Sp7	TCTCTGCTTGAGGAAGAAGCTC	TTGAGAAGGGAGCTGGGTAG
Runx2	AGTCAGATTACAGATCCCAGGC	GCAGTGTCATCATCTGAAATACG
Spp1	AGCAAGAAACTCTTCCAAGCA	TGGCATCAGGATACTGTTCATC
Twist1	GCTCAGCTACGCCTTCTCC	ACAATGACATCTAGGTCTCCGG
Actb	GCCACCAGTTCGCCAT	CATCACACCCTGGTGCCTA
Pgk1	GAGCCCATAGCTCCATGGT	ACTTTAGCGCCTCCCAAGA

Table 3. qRT-PCR primer for the cell culture system.

- 384-well plates (ABgene / TF-0384)
- Optical PCR protective film – Optical Adhesive Covers (Applied Biosystems / 4311971)
- Taq-Man 7900HT Fast Real-Time PCR System (Applied Biosystems) including software (Applied Biosystems ABIprism SDS 2.1)

6.2. Measurement day

- 24-well Primaria™ cell culture plate
- The samples (n wells) of both biological groups are pooled for the preparation (no technical replicates)
    - 6 x wt → 4 wells are pooled for wt sample
    - 6 x mut → 4 wells are pooled for mut sample

- RNA isolation is performed using the RNeasy Mini Kit according to the protocol "Purification of Total RNA from Animal Cells Using Spin Technology"
    - 10 µl β-mercaptoethanol is added to 1ml of buffer RLT

- aspiration of old media from the wells
- cells are washed once with 1x PBS
- for each group (6 wells) the RNA of 4 wells is pooled: 350 µl buffer RLT is added to well 1 and afterwards transferred to well 2, well 3 and well 4 for cell lysis (repeated up and down pipetting of the buffer should be performed to ensure complete lysis of the cells)
- the lysate of each pooled sample (350 µl) is loaded onto a QIAshredder column and centrifuged for 2 min / RT / 13000 rpm to homogenize the lysate
- both homogenized lysates (1 x wt / 1 x mut) are stored at -80°C

6.3. Assay analysis

- RNA isolation
  - RNA isolation is completed with all frozen samples (lysates) according to the protocol (5 x wt / 5 x mut)
  - RNA is eluted in 50 µl RNAse free H_2O and stored at -80°C

- cDNA synthesis
  - for each sample the RNA is reverse transcribed into cDNA according to the following scheme

RNA	15 µl
Primer OligodT	2 µl
→ **Pre-annealing**	**10 min / 65°C**
5x buffer	10 µl
dNTP-Mix	2.5 µl
RNAse Out	2 µl
Super Script II	2 µl
H_2O	16.5 µl
→ **Incubation**	**60 min / 42°C**
→ 50µl cDNA	(stored at -80°C)

- qPCR reaction / master mix
  - for each sample a qPCR reaction is performed as described below
  - the cDNA is applied in a 1:10 dilution with H_2O
  - each sample is analyzed with 4 technical replicates for the expression of 15 genes (overall 60 qPCR reactions for each sample) – therefore a master mix of 65x is recommended for each sample
  - please note: the forward and reverse primer for each gene are combined in one mixture with 5 µM each, the primers are not included in the master mix but pipetted separately

Reagents	**qPCR reaction 1x**	**qPCR master mix (65x)**
2x Power Sybr® Green	10 µl	650 µl
cDNA (1:10)	2 µl	130 µl
H_2O	6 µl	390 µl
Primer fw + rv (5µM each)	2 µl	/
	→ 20 µl	→ 1170 µl

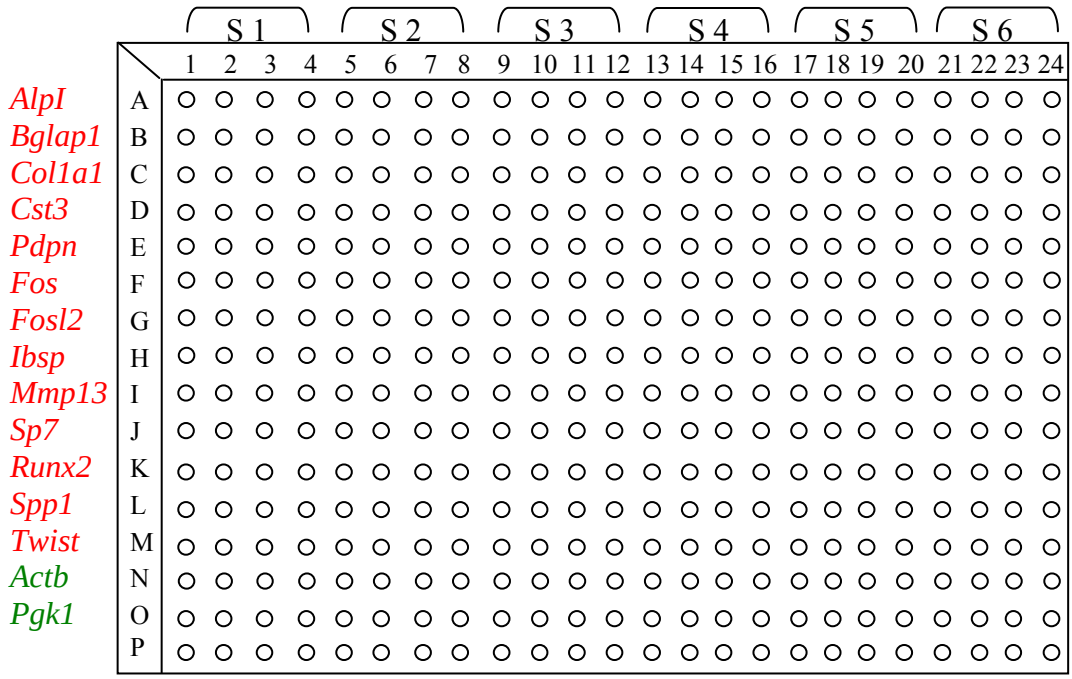
- Expression analysis with Taq-Man 7900HT
  - the 15 genes that are analyzed in the expression analysis are classified in 2 groups:

    1) target genes (target) (13) - bone relevant marker genes, expression is compared between the different samples
    2) reference genes (reference) (2) - internal reference for the target genes in each probe (housekeeping genes)

  - the qPCR reactions are pipetted in 384well plates and the expression analysis is performed using the Taq-Man 7900HT
  - given the huge number of samples, genes to be analyzed and technical replicates (10 samples x 15 genes x 4 replicates = 600 reactions overall), two plates have to be applied with the following distribution of the samples

Plate 1	Plate 2
wt T0	wt T15
mut T0	mut T15
wt T3	wt T21
mut T3	mut T21
wt T9	/
mut T9	/

  - the samples (master mix) and primers are pipetted in a 384well plate according to the following scheme (red - target genes / green - reference genes)



Primers are described by the name of the respective genes and the samples are labeled with S1 - S6. As depicted, each sample is analyzed with 4 technical replicates for the expression of each gene (S1 = 1/2/3/4, S2 = 5/6/7/8 …).

- the qPCR reactions (master mix and primer) are pipetted in the 384well plate using an adjustable Matrix 8-channel pipette
- primers are added first with 2 µl per well according to the scheme and afterwards the master mixes are added with 18 µl per well
- the plate is sealed with the Optical Adhesive Covers and centrifuged for 2 min / RT / 2000 rpm
- the qPCR is performed in the Taq-Man 7900HT according to the following scheme and the results are saved as SDS file

Temperature	Time	Cycles
95°C	10 min	1
95°C	15 sec	40
60°C	1 min	
Melting curve (55°C – 95°C)		1

- Evaluation of the qPCR and determination of the crossing point (C_t)
  - the saved SDS files are analyzed using the software ABIPrism SDS 2.1
    - File → Open
    - Analysis → Analysis Settings… → Automatic C_t √
    - Analysis → Analyze
    1) melting curve of each qPCR has to be checked
    2) C_t-values are exported: File → Export → save as Text file

- Determination of target gene expression
  - C_t-values are transferred from the Text file into Excel file
  - <u>for each sample</u> the determination of the expression values of the 13 target genes is performed according to the following procedure:

    1) the C_t values of the 4 technical replicates ($C_{t1/2/3/4}$) from each gene (13 x target and 2 x reference) are averaged to obtain the arithmetic mean C_t for each gene (please note: $C_{t1/2/3/4}$ values with a difference of ± 0.5 from the mean value are not considered for the calculation)

$$C_t = \frac{(C_{t_1} + C_{t_2} + C_{t_3} + C_{t_4}}{4}$$

    2) the C_t value of both housekeeping genes *Actb* (C_{tActb}) and *Pgk1* (C_{tPgk1}) is averaged to obtain the reference C_t value (C_t reference) for the determination of the target gene expression

$$C_{t\ \text{reference}} = \frac{(C_{t\ \text{Actb}} + C_{t\ \text{Pgk1}})}{2}$$

3) the following equation is used to calculate the expression for each of the 13 target genes (E_{target})

$$E_{target} = \frac{2^{C_t\ reference}}{2^{C_t\ target}} \times 100$$

4) E_{target} corresponds to the expression level of the respective target gene in percentage to the reference value (expression of *Actb* and *Pgk1* = 100%) in the analyzed sample

e.g.: expression of *AlpI* in a sample xy (for instance T3 wt)

$$C_t\ reference = \frac{C_t\ (Actb + Pgk1)}{2} = 18.2$$

$$C_t\ target = C_t\ AlpI = 19.7$$

$$E_{target} = \frac{2^{C_t\ reference}}{2^{C_t\ target}} \times 100 = E_{AlpI} = \frac{2^{18.2}}{2^{19.7}} \times 100 = \underline{\underline{35.36}}$$

→ in the sample T3 wt, the expression of the *AlpI* gene equates 35.36% compared to the expression of the reference value (*Actb* and *Pgk1* = 100%)

5) for each of the 10 samples the expression values for all 13 target genes is determined and saved (Excel file)

6.4. Results and presentation

- For each sample (biological group at certain measurement day) the expression level of the 13 target genes is determined in [%] to the expression of the reference
- For graphic presentation of the gene expression during the culture period a single diagram for each of the 13 target genes is drawn with the expression level of the respective gene on the y-axis against the measurement days on the x-axis for both groups (wt and mut)