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The dependence of autologous chondrocyte transplantation on varying cellular passage, yield and culture duration

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1. Introduction

Peterson and Brittberg pioneered clinical autologous chondrocyte implantation (ACI) in 1994 [1]. Nowadays, the technique is accepted for treatment of large diameter hyaline cartilage defects across the knee joint [2], currently providing with satisfying long-term data [3]. The technique has been and currently is experiencing constant modifications. Frequent revision surgery due to periostal hyperthrophy and shorter operating time guided modifications in the form of replacing the periost (ACI-P) by an artificial membrane (ACI-C). Varying input such as better handling, potential *in vitro* cellular re-differentiation, initial stability or homogenous cell distribution resulted in the most recent technical diversification: matrix-assisted chondrocyte transplantation (m-ACI). Chondrocytes are seeded within a 3-D matrix to be subsequently replanted. Marlovits [4] classified into first (ACI-P), second (ACI-C) and third (m-ACI) generation chondrocyte transplantation. All of which are in current worldwide use with multiple competing industrial products being available for the treating physician. The practical realization of neither approach is clearly standardized, which is especially true for the parameters of how often to passage the cells *in vitro*, how many cells to transplant, and for which time

Abbreviations: CMC, cell-matrix-construct; CMC-i, cell-matrix-construct harvested after *in vitro* culture; CMC-e, cell-matrix-construct harvested after *in vivo* exposure; C, cell yield, number of chondrcytes seeded within the matrix; P, cell passage, number of cell passsages prior to seeding of the matrix; T, culture duration, time that cell-seeded membranes remained within *in vitro* culture until retransplantation.

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span to culture the cells on the membrane (in case of m-ACI) until retransplantation. In parallel, perpetual novel resembling techniques to transplant articular chondrocytes are being invented even though basic tissue engineering aspects have yet not been fully inspected. Eventually, randomized controlled trials have shown both, ACI superiority or no difference, when comparing with the much less elaborate technique of microfracturing [5,6]. On the contrary, the clinical outcome of different ACI generations has been very infrequently compared, while just previous studies have shown no clear difference [7–9]. Picturing clinical inconclusion [10], there is constantly increasing data influx from cartilage experiments [11]. However, even though the parameters of cell count [12], passage [13] as well as membrane-holding time [14] have been inspected in isolation, their potential combinative effects on the technique of matrix-assisted autologous chondrocyte transplantation have not been analyzed before. It defines the aim of this animal study.

2. Materials and methods

2.1. Study design

The intention of this study was to investigate on the effects of the cell culture parameters passage, cell yield, membrane-holding time on gene expression levels of rabbit articular chondrocytes cultured within 3-D matrices mimicking matrix-assisted chondrocyte transplantation techniques. By use of a "duplicate model", we intended to recognize in which manner familiar data from the *in vitro* cell-matrix-constructs devise through 12 weeks of *in vivo* exposure.

A total of 60 animals (New Zealand White Rabbits) were applied for this study and processed as shown in Table 1. Cartilage biopsy was performed in 54 animals (right knee) and 6 animals served as control. Cartilage cells were isolated and then proliferated *in vitro* (see below). Thereby cell preparations (*in vitro* cell culture parameters) were divided according to the different study groups ($n = 18$), which were defined by:

- Three varying cell **Passages (P)** (Passage 1 = P1; Passage 3 = P3; Passage 5 = P5);
- Three different **Cell yields** to be cultured on the matrix **(C)** (2×10^5 = C1; 1×10^6 = C2; 3×10^6 = C3);
- Two different **Membrane-holding times** (total duration the cells remained on the matrix) *in vitro* prior to retransplantation **(T)**(5 h = T1; 2 weeks = T2).

Each time, per animal, a total of 4 cell-laden matrices (chondrocyte-matrix-construct = CMC) were assembled from the initial cartilage biopsy whereof two were identical according to P, C and T. From those 4 membranes the duplicates were divided and two (from different study groups) were re-implanted into two standardized trochlear defects of the biopsy contralateral (left) knee joint (CMC-e; *e = explant*). The remaining two membranes (again from different study groups) were harvested (CMC-i; *i = in vitro*) isochronous to implantation of the other 2 duplicate membranes. Per study group ($n = 18$), there were a total of $n = 12$ membranes (overall total of $n = 216$ cell-seeded membranes; $n = 108$ for implantation *in vivo* and $n = 108$ for direct *in vitro* analysis). After 12 weeks *in vivo*, animals were euthanasised and CMC-e regenerates were as well harvested. A total of 6 animals (12 artificial defects) were used for control. 6 defects remained empty and 6 defects were treated with an unseeded (no cells) membrane which had been cultured for either 5 h ($n = 3$) or for 2 weeks ($n = 3$) in accordance to the cell-seeded membranes.

2.2. Animal model, cell culture and matrix preparation

The procedures involving animal care and treatment were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (DIRECTIVE 86/609/EEC; German animal welfare law; FELASA guidelines). The protocol was approved by the district government of upper bavaria (file number 55.2-1-54-2531-65-07). Female New Zealand White Rabbits (Crl:KBL(NZW)) were obtained from Charles River (Kisslegg, Germany). The animals were kept under conventional keeping conditions and controlled room temperature (19 °C ± 2 °C). Acclimatization lasted 7 days. The animals were housed in cages with water and food ad libitum. Lighting was on a 12 h a day on/off cycle. By the time of treatment, the animals had reached a bodyweight of approximately 3.5 kg with closed growth plates. Initially, a standardized large cartilage biopsy out of the right knee was taken in every animal. Anesthesia was induced by an intravenous injection of 10 mg/kg propofol and maintained after intubation with 1.5 mg/kg/h propofol and 0.05 mg/kg fentanyl intravenously. The rabbits were ventilated with 100% oxygen, intraoperative antibiosis was achieved by i.v. 0.5 ml Borgal® 24% (Veterinaria AG, Zurich, Switzerland). After shaving and desinfection, the right knee joint was opened by a medial parapatellar arthrotomy under sterile conditions and the patella was laterally displaced. Hereafter, full-thickness cartilage pieces were peeled out of the trochlea and proximal femora with a scalpel and the joint was closed in layers. For postoperative analgesia the next 3 days 4 mg/kg carprofen was administered once a day subcutaneously plus 0.03 mg/kg buprenorphine subcutaneously twice a day. Articular chondrocytes were further processed as previously described in Ref. [15]. The initial biopsy regularly gave $2.5–3.0 \times 10^5$ cells. For that, passage 1 was used to directly seed the cells on the matrix, while P3 and P5 resulted in 2–3 and 4–5 population doublings, respectively. When the varying passages and cell quantities were achieved, chondrocytes were trypsinized and the cell pellet was condensed in a total of 12–20 μl (depending on cell quantity) cell culture medium (complete saturation of the membrane was achieved) and passively seeded onto the porous, cell-adhesive side of the bilayer collagen I/III scaffold (Chondro-Gide®, Geistlich Pharma AG, Wolhusen, Switzerland), which was cut to the exact dimensions of the trochlear defect (see below). Sequential pre-test evaluations (histology, data not shown) demonstrated even cellular distribution across the complete membrane with no leakage during further processing. Cell-matrix-constructs were then placed in specific culturing containers to deter cell migration onto the dish bottom and therefore inhibit cellular migration off the matrix. Cell-seeded membranes were left without further cell culture medium within an incubator for 1 h for safe adherence of the chondrocytes. After that, cell-matrix-constructs were placed within new culturing containers containing fresh medium. Care was taken to not wash out any cells off the membranes even though strong adherence is expected. Hereafter, CMCs were either replanted consecutively after a total of 5 h *in vitro* (T1) or left under standard culturing conditions for 2 weeks (T2) to create the 2 different membrane-holding times. For CMC implantation, the rabbits were anesthetized again as described above. A medial parapatellar arthrotomy was performed on the left knee and a custom-made drill (4.0 mm in diameter) with an automated drill stop was used to create 2 isolated chondral defects within the trochlea groove. No opening of the subchondral plate or bleeding was generated. The defects were rinsed with sterile saline, then 2 autologous CMC products from different study groups were press-fit implanted (porous side facing defect bottom) into the defects flush to the surrounding surface and sealed with fibrin glue to assure certain fixation. After clotting, the patella was relocated and full range of motion was applied to the joint 10 times. The patella was again displaced and membranes were checked for any event or sign of instable fixation. Hereafter, the patella was again replaced and the joint closed in layers. Early euthanasia 2 weeks postoperative implantation (pretests) revealed no membrane delamination following animal mobilization. Postoperative analgesia was given as described above. Generally, animals applied full weight-bearing from day one postoperative.

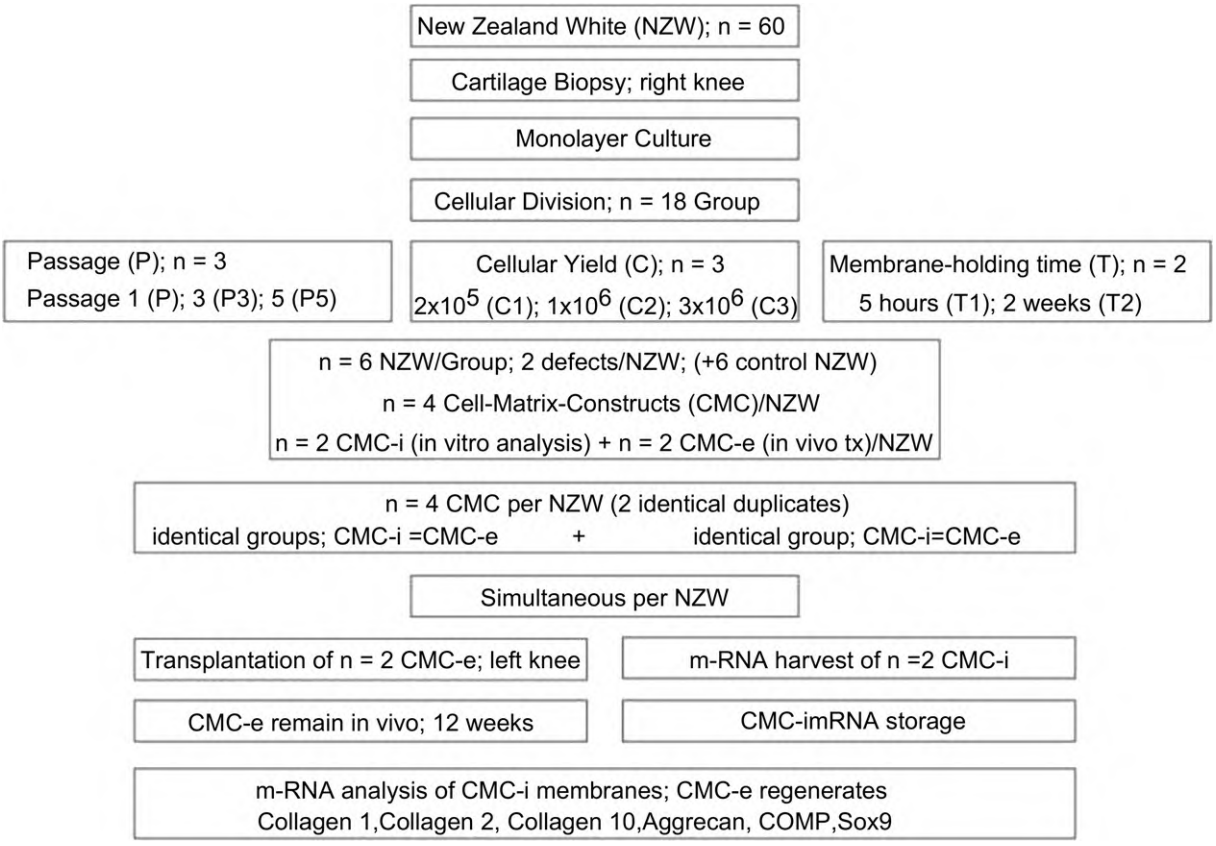
2.3. RNA extraction and analysis

Total RNA was isolated using the NucleoSpin RNA II® kit (Macherey–Nagel, Düren, Germany) following the manufacturer's protocol for total RNA purification from cultured cells and quantified utilizing the NanoDrop ND-1000® spectrophotometer (PEQLAB, Erlangen, Germany). For quantitative polymerase chain reaction (qRT-PCR) expression profiles were assessed via one-step qPCR on a Rotor-Gene-6000® (Corbett Life Science, Qiagen, Hilden, Germany) using the Quanti-Tect® SYBR® Green RT-PCR kit (Qiagen, Hilden, Germany) followed by an specific melting-curve analysis of each assay. Primer-3 software was utilized for primer design [16]. The primer sequences were Aggrecan, 5′- CTA CGA CGC CAT CTG CTA CA -3′ (forward), 5′- TCT TCA GTC CCG TTC TCC AC -3′ (reverse); Collagen 2, 5′- GTC CCT CTG GCA AAG ACG -3′ (forward), 5′- GCC CTT CTC TCT CTG GCC TA -3′ (reverse); COMP, 5′- GGA ACC CAG ACC AGC GTA AC -3′ (forward), 5′- GTC CGC GTT GCT CTT CTG -3′ (reverse); SOX9, 5′- AAG ATG ACC GAC GAG C -3′ (forward), 5′- CAG CGT CCA GTC GTA G -3′ (reverse); Collagen 1, 5′- CTG CAA GAA CAG CAT TGC AT -3′ (forward), 5′- GGC CAA CGT CCA CAT AGA AT -3′ (reverse); Collagen 10, 5′- GCT TAC CCA GCG GTA G -3′ (forward), 5′- CTC CCT GAA GCC TGA T -3′ (reverse); β-Actin, 5′- CAG CGG AAC CGC TCA TTG CCA ATG G -3′ (forward), 5′- TCA CCC ACA CTG TGC CCA TCT ACG A -3′ (reverse). 1.0 μl mRNA template, 0.1 μl RT-Mix, 5.0 μl 2xQuanti-Tect® SYBR® Green RT-PCR Master Mix, 0.4 μl of each primer (forward, reverse) and 3.1 μl RNase-free water composing a 10 μl reaction mixture. Thermocycler settings consisted of a reverse-transcription step at 50 °C (30 min), activation of the polymerase reaction at 95 °C (15 min), followed by 38 cycles of denaturation at 94 °C (15 s), annealing at 58/60/62 °C according to primer specific requirements (30 s), elongation at 72 °C (30 s) and fluorescence detection at 80 °C (15 s). β-Actin and HPRT-1 were used as internal controls. All reactions were performed in duplicate setup followed by a melting-curve analysis for each qRT-PCR run. Expression levels were calculated using the comparative CT method [17]. Collagen 2, Aggrecan, COMP and Sox9 are regarded differentiation targets. Collagen 1 and Collagen 10 are regarded dedifferentiation and hypertrophy targets.

2.4. Statistical analysis

Statistical analysis was performed using the software package SPSS (Version 17; SPSS Inc., Chicago, IL). Skewed data distribution was normalized by applying natural logarithm transformation. Linear mixed regression models were used for the analysis of quantitative parameters. In this term, marginal means for the factor variables

Table 1
Study Design. A total of 60 New Zealand White Rabbits were applied. Chondrocytes were divided into 18 different groups according to varying passages, cellular quantity and time on the membrane. Each time, per animal, a total of 4 membranes were designed whereof 2 were identical according to passage (P), quantity (C) and time on membrane (T). Duplicates were split and simultaneously 2 (ergo from different groups) were re-transplanted (tx) and 2 were harvested from *in vitro* culture to isolate mRNA. Transplanted membranes were harvested after 12 weeks *in vivo* and mRNA was analyzed from *in vitro* and *in vivo* membranes.



P, C and T were calculated. In a further detailed analysis, post-hoc comparisons of factor-level combinations ($3 \times 3 \times 2$) were conducted, depending on previous (overall) significance testing. Group comparisons noted within results section are, if not ascribed differently, all significant at a two-sided 0.05 level of significance. To retain a maximum of power within the multiple comparisons being conducted in this explorative study, no adjustment of alpha-error level was conducted. For the purpose of graphical visualization only the parameters P and T were applied since the mathematical impact of C was generally less effective when comparing to P and T.

3. Results

3.1. Differentiation

There was a highly significant proportional linearity between the respective CMC-i and the consecutive CMC-e values (Fig. 1). CMC-i values were generally (exceptions apply, see below) significantly higher than CMC-e values. Mathematically isolating P, C and T it appeared that increasing passage (P) typically decreased expression *in vitro*. Resulting varying expression amplitudes related to shifting passages *in vitro* tended to level *in vivo*, while the given trend and evident differences remained equal. With increasing cellular yield (C) on the matrix, expression not-significantly decreased within CMC-i products where C1 was usually more/as effective as C2 and both more effective than C3. *In vivo*, this trend shifted to C2 and C3 membrane quantities with a clear superiority of C2. T1 combinations had significantly stronger *in vitro* as well as *in vivo* expression than comparable T2 combinations. Estimation of

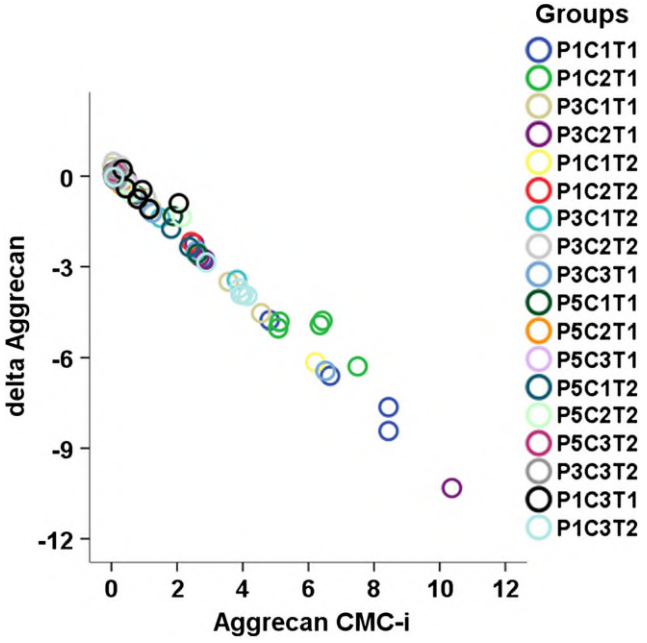


Fig. 1. Exemplary (for differentiation targets) visualization of the linearity between CMC-i and delta (the mathematical difference of CMC-e minus CMC-i) Aggrecan values of all possible P, C, T groupings ($n = 18$).

fixed effects highlighted that the parameters P and T comparably affected the respective gene expression levels (with a trend for more impact of T), while C had less effects. Passage 1 cells combined with a short holding time on the matrix resulted in an overall optimal expression. This finding remained for *in vitro* expression, while generally *in vivo* T2 times were superior over T1 times when cells were passaged three times. Among all differentiation targets (cumulative value; ranking order) the cell culture combination of P1C2T1 resulted in the overall strongest expression within CMC-i as well as CMC-e combinations.

3.2. Collagen 2

The expression of CMC-i products was generally higher than the expression of CMC-e products. Cell-matrix combinations with P1 or P3 cells in combination with T1 holding periods constantly had very high CMC-i expression (comparing to overall mean) which dropped to lower values at the time of explantation (Fig. 2). In contrast, cell-matrix combinations with P5 cells and/or T2 holding periods commonly had very low CMC-i values, which in turn increased to significantly higher levels at the time of explantation (compared to CMC-i). Typically, the animals decreased an initially high CMC-i expression, maintained an average CMC-i expression and increased a below average CMC-i expression during the time course *in vivo*. Lower cell quantity (C1 and C2) had commonly equal as well as respective higher expression than highest (C3) yield on the membrane *in vitro*. *In vivo*, collagen 2 expression was significantly higher in C2 than in C1, which in turn was higher than in C3. While *in vitro* T1 combinations were persistently higher than T2 ones, this trend changed to superior T2 combinations when paired with P3 or P5 cells *in vivo*. Clearly strongest expression was achieved within P1C1T1 as well as P1C2T1 among CMC-i combinations, while the combinations of P1C3T1 and P1C2T1 resulted in the strongest expression among CMC-e products. P3C2T1 was overall third *in vitro* while P3C2T2 was overall third in *vivo*; both with approximately half the expression of the former combinations.

3.3. Aggrecan

Paralleling collagen 2 mRNA the expression of CMC-i products was higher than the expression of CMC-e products (Fig. 2). Without exception, the expression significantly decreased from *in vitro* to *in vivo* values. Cell-matrix combinations with P1 or P3 cells in combination with T1 holding periods constantly had highest CMC-i expression. Lower cell quantity (C1 and C2) had commonly equal as well as respective higher expression than highest (C3) yield on the membrane *in vitro*. *In vivo*, aggrecan expression was significantly higher in C2 than in C1, which was equal to C3. *In vitro*, T1 combinations were persistently higher than T2 ones, this trend changed to superior T2 combinations when paired with P3 or P5 cells *in vivo* while these *in vivo* differences were not significant anymore. Clearly strongest expression was within P1C1T1 as well as P1C2T1 within CMC-i products, while it was strongest among P1C2T1 *in vivo*.

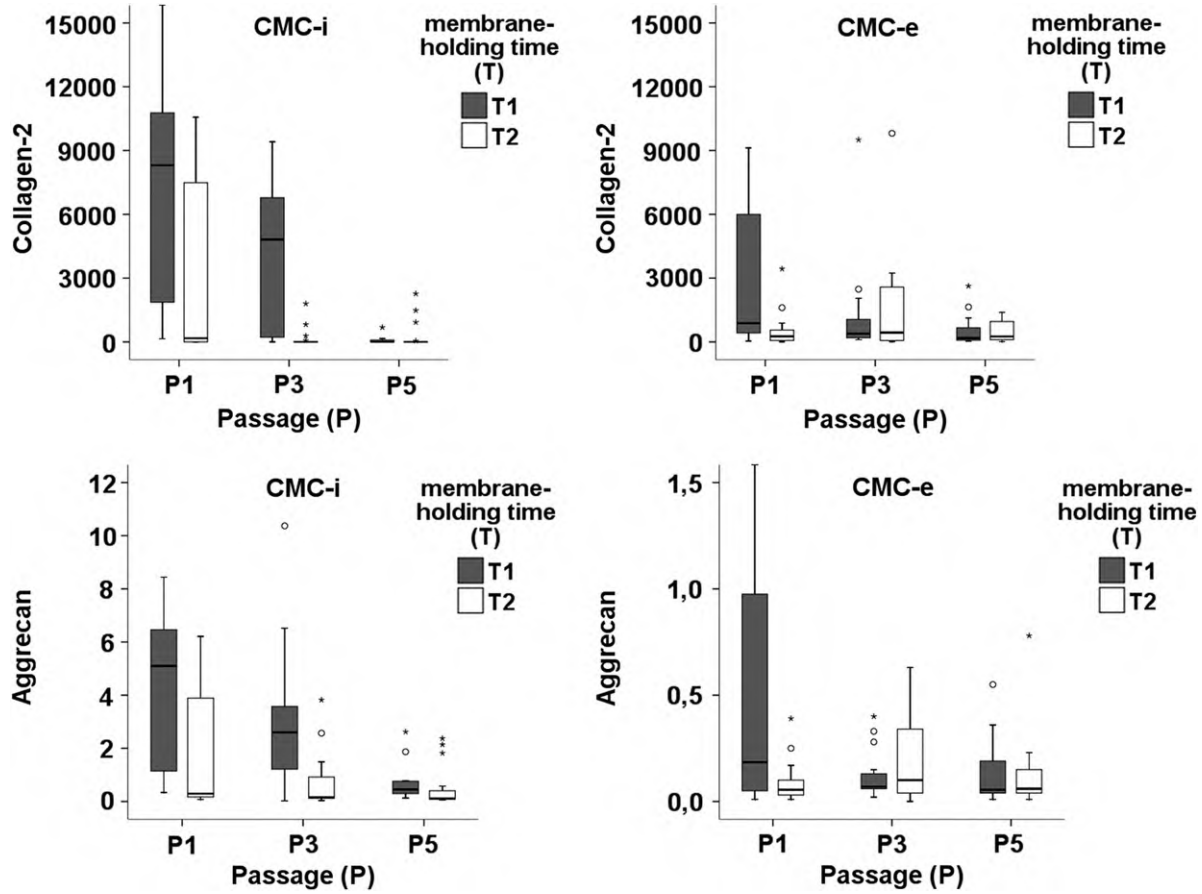


Fig. 2. Collagen 2 and Aggrecan mRNA expression within *in vitro* membranes (CMC-i) and *in vivo* regenerates (CMC-e) subdivided by passage 1 (P1), 3 (P3), 5 (P5) and membrane-holding time 5 h (T1), 2 weeks (T2).

3.4. COMP

Comparably to collagen 2 and aggrecan, CMC-i values were higher than CMC-e values, while the difference in expression was much less distinct (Fig. 3). In contrast to the former genes, combinations of low P and T1 demonstrated an induction of COMP expression after 12 weeks *in vivo*. Furthermore, *in vitro* values were constantly lower among T1 assemblies when compared to T2. This effect condensed after 12 weeks of *in vivo* exposure. Cellular quantity on the membrane had no significant impact on neither *in vitro* nor *in vivo* expression. No specific CMC assembly stood out strongly significant.

3.5. Sox9

CMC-i values for all groups were higher compared to CMC-e values (Fig. 3). Generally, T1 values were higher than T2 values. Among T1-CMC-i passage 3 resulted in significantly higher Sox9 expression than P1, while there was no difference between P3 and P5. Among T2-CMC-i passage 1 had stronger expression than P3, which again had stronger expression than P5. In contrast to the previous targets higher cellular density resulted in significantly stronger expression. Strongest overall expression was within P3C3T1 among CMC-i and within P1C2T1 and P1C3T1 among CMC-e products.

3.6. Dedifferentiation and hypertrophy

In general, dedifferentiation expression, in particular collagen 1, resulted in less shifting amplitudes in relation to varying P, C and T when compared to the differentiation targets. There was as well significant linearity for Collagen 1, while this analysis was not significant for Collagen 10. In contrast to the differentiation targets, CMC-i values were significantly lower in expression than CMC-e values. This induction during 12 weeks *in vivo* was much stronger for the expression of collagen 10. Also in contrast to differentiation targets, a varying category C had much stronger effects on the expression of dedifferentiation targets.

3.7. Collagen 1

The expression increased with gradient passages (Fig. 4). Differing cellular yield did not significantly affect the outcome, while higher membrane density resulted in lower collagen 1 expression. Prolonged matrix-holding time not-significantly increased collagen 1 expression. There was a general trend for strongest expression among T2 combined with P3 or P5 and C1 or C2 cellular yield. Among *in vivo* products, the expression increased with gradient passages. C2 cell count on the matrix resulted in the lowest collagen 1 expression, while the expression in C1 was higher than in C3. No significant expression difference remained among varying T *in vivo*. Overall strongest expression was among T2 combined with P3, P5 products.

3.8. Collagen 10

Varying passages were not significantly different while strongest expression was within P3 combinations *in vitro* (Fig. 4). Lowest expression was within C2 products, which increased among C1 and

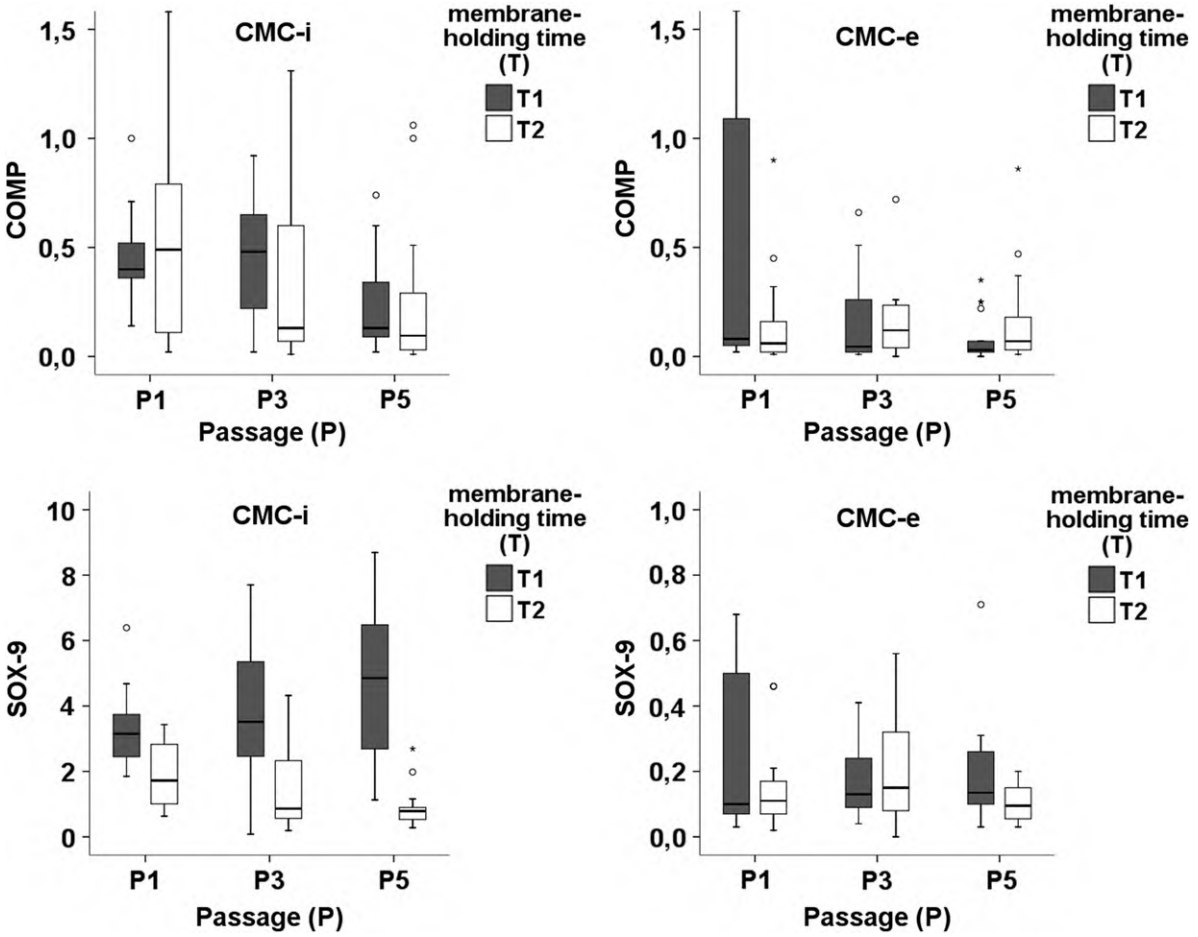


Fig. 3. COMP and Sox9 mRNA expression within *in vitro* membranes (CMC-i) and *in vivo* regenerates (CMC-e) subdivided by passage 1 (P1), 3 (P3), 5 (P5) and membrane-holding time 5 h (T1), 2 weeks (T2).

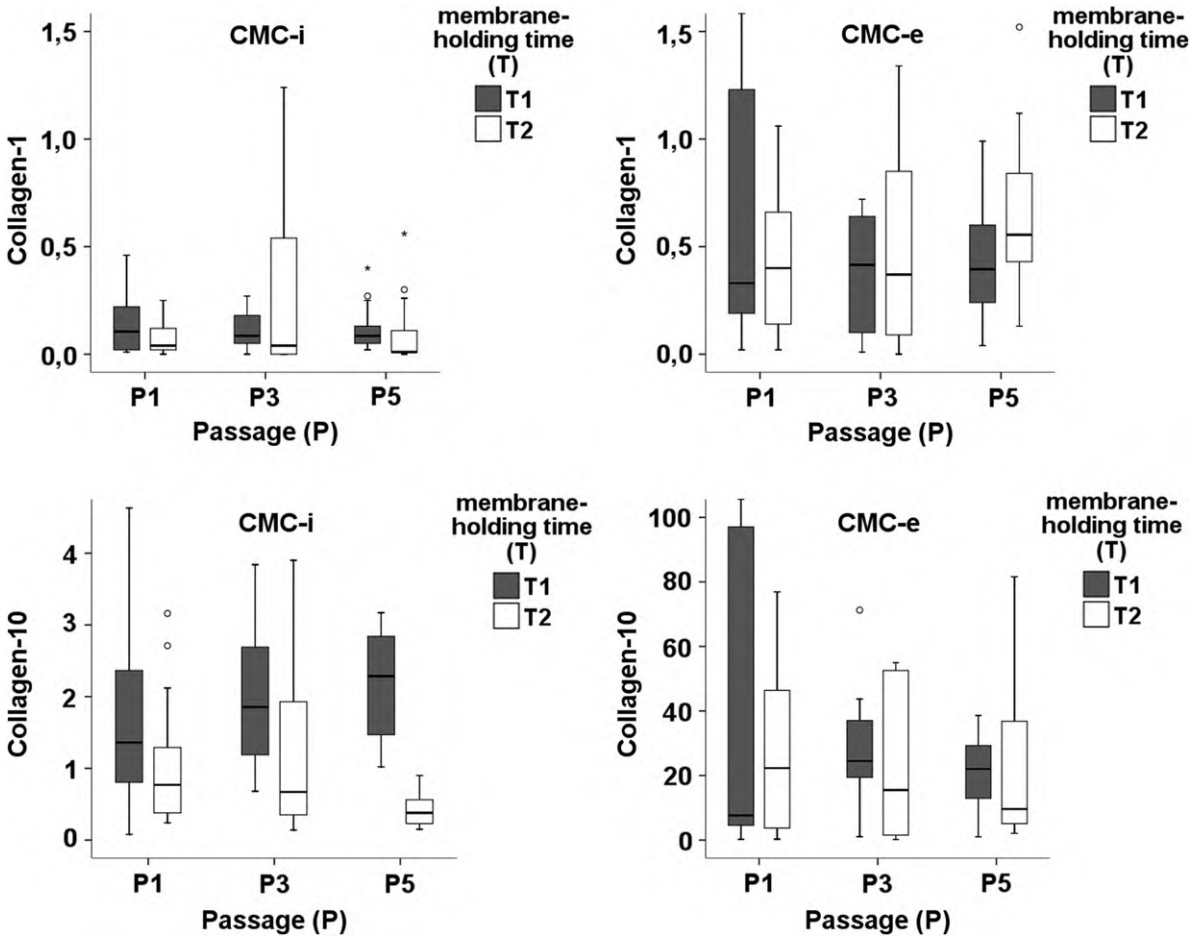


Fig. 4. Collagen 1 and Collagen 10 mRNA expression within *in vitro* membranes (CMC-i) and *in vivo* regenerates (CMC-e) subdivided by passage 1 (P1), 3 (P3), 5 (P5) and membrane-holding time 5 h (T1), 2 weeks (T2).

was highest within C3 CMC *in vitro*. T2 had significantly lower expression than T1. There was an inverse expression behavior dividing T1 and T2 products. Collagen 10 expression increased among T1 CMC with gradient P, while the opposite was true for T2 composites. *In vivo*, higher P and T resulted in decreased expression, while the opposite was true for varying C constellations. Strongest expression was among P1T1 *in vivo*, while there was no significant differences among varying *in vitro* products.

3.9. Control

mRNA quantification remained under detection limits for all CMC-i compilations. The same was true for unseeded CMC-e regenerates as well as empty defects, while generally streaks of collagen 1 and/or collagen 10 were detected. Collagen 2, Aggrecan, COMP or Sox9 was never explored in any control by application of the study detection limit (data not shown).

4. Discussion

Results of this study display that changing culture parameters result in a significantly different outcome *in vitro* and *in vivo*, which are linearilly connected when single gene expression is concerned. It gives the potential ability to predict the *in vivo* outcome based on the established *in vitro* data. While the parameter C had clearly less impact when compared to P and T, a combination of P1 with T1 generally resulted in an optimal chondrogenic expression *in vitro* and *in vivo*. This trend divided at passage 3 where *in vitro* T1-membranes remained superior over T2-membranes with converse information *in vivo*. Regarding an optimal *in vivo* chondrogenic differentiation, the combination of 1×10^6 Passage 1 chondrocytes precultured within the membrane for 5 h (P1C1T1) resulted in an ideal expression pattern.

There exist imprecise guidelines for the realization of ACI aiming to fulfill the cornerstones quality, efficacy, safety when specific culturing aspects are concerned. Every autologous chondrocyte transplantation is arranged by a cartilage biopsy, which is subsequently send to a laboratory for further processing. In order to limit donor-site morbidity, currently, large quantities of chondrocytes are required and therefore generated *in vitro* to realize ACI. This can only be accomplished by multiple population doubling procedures automatically entailing the process in which a chondrocyte's phenotype changes from chondrocytic to fibroblastic termed dedifferentiation [18]. It remains an unmatched goal to obtain both proliferation and differentiation, which is related to the simple fact that proliferation is antagonistic to differentiation [19,20]. The currently youngest ACI generation is matrix-associated while upcoming, concurring products such as ACT-Cs [21], bioreactor assembled cartilage [22], or minced cartilage [23] produce a large spectrum of different techniques to apply. This engineering evolution is taking place, even though, particular general aspects of chondrocyte transplantation have not been defined yet and are not precisely guideline-regulated. Those are: how many cells should be transplanted per defect volume (this value is usually given per

square centimeter even though it is known that cartilage thickness clearly differs across the knee joint [24,25]); how often should the cells be passaged *in vitro* prior to implantation (and how many population doublings are being performed); and (if applicable) how long should the cells be cultured on a respective matrix prior to implantation. Based on existing knowledge, one may hypothesize that an optimal composition of these parameters can enhance cellular quality with regard to chondrogenic phenotypic (y). It is an upcoming issue when ACI is concerned [6,26] and may impact the clinical outcome.

Already in 2001, Dell'Accio demonstrated, using freshly isolated human articular chondrocytes injected into nude mice, that resistance to mineralization, vascular invasion, and replacement by bone was positively associated with the expression of fibroblast growth factor receptor 3, bone morphogenetic protein 2, and collagen 2 [27]. In continuation, Schmal and coworkers just previously demonstrated that the clinical outcome following cartilage repair procedures is significantly correlated to synovial expression patterns, which therefore seem to play an important role in surgically induced cartilage repair [28]. Subsequently, Niemeyer et al. (in press) reported that the clinical outcome following knee ACI was significantly (chronologically) related to the initial (at the time of transplantation) cellular expression of CD-44 and Collagen 2 clearly underlining previous outcome data demonstrated by Dell'Accio and Schmal. There is evidence to suggest that cellular quality (in particular collagen 2) may play a pivotal role to form stable and pure cartilage *in vivo* holding the capacity to ensure a satisfying clinical outcome. In promotion of this suggestion, Brun and colleagues [29] noted a correlation between the symptomatology of patients and the nature of the reparative tissue using histologic sections taken from patients during second-look arthroscopy following m-ACI for cartilage defect repair. Asymptomatic patients developed predominantly hyaline tissue in a highly significant percentage of cases, while symptomatic patients contained mostly fibrocartilage or mixed tissue. Definition of the term hyaline cartilage was, again, strongly dependent on positive collagen type 2 immunohistochemistry.

Paralleling this previous information the expression of collagen 2, in our experiment, was considerably affected by variation of the determined cell culture parameters and resulted in significantly shifting amplitudes among the different study groups. The single factor passage had strong influence on the varying expression with a clear trend for optimal differentiation after one/few passages. Linear mixed regression exposed that with increasing passage the expression of differentiation targets decreased *in vitro* and *in vivo* (data not shown). This information parallels data from Kang and colleagues [30] who compared the growth rate, viability, collagen 1/2 synthesis, and apoptotic activity of New Zealand White knee articular chondrocytes with passage number of 1, 2 and 5 *in vitro*. With increasing passage number cell growth rate and viability decreased whereas apoptotic cells increased. Passage 2 chondrocytes exhibited a high expression of collagen 2 and a low expression of collagen 1, while in contrast, passage 5 chondrocytes exhibited the opposite, indicating chondrocyte dedifferentiation. After implantation of cell-seeded scaffolds into subcutaneous spaces of athymic mice microscopic analyses revealed mature and well-formed cartilage within passage 1 products, while passage 5 chondrocytes did not have chondrocyte morphology or cartilage-specific extracellular matrices. Repeat population doubling or passaging procedures, depending on the cell culture technique applied, has to be done to improve cellular quantity. It exists the persistent belief that clinically 1 million cells have to be transplanted per square centimeter even though clear scientific back-up is missing.

Our results indicate that quantity had least impact when compared to T and P. While the expression of differentiation markers continuously decreased with increasing C (linear mixed regression, data not shown) *in vitro* (with the exception of Sox9), strongest expression was achieved among C2 membranes *in vivo*. Literature data on matrix density are conflicting. Willers and colleagues did not find any significant difference after conducting an animal m-ACI dose–response study, using New Zealand White knee articular chondrocytes at various cell densities between 10^4 and 10^6 cells/cm^2 defect [31]. Contrary to that, Concaro reported on improved cellular (human chondrocytes) quality of *in vitro* tissue engineered constructs with increasing chondrocyte density cultured for 14 or 28 days on chitosan scaffolds [12]. Khoshfetrat [32] showed that high rabbit chondrocyte cell density (compared to low density) within 3-D surroundings *in vitro* significantly decreased migration while synchronous to that the expression in differentiation targets such as collagen 2 was upregulated. Furthermore, there was no significant difference in the collagen 1 expression in dependence of varying density, which is in line with our data that are in general display of low collagen 1 expression. Only moderate collagen 1 expression amplitudes have been comparably reported within previous studies as well using animal cells [34,38,44,45]. Yet, increased expression of collagen 10 as a sign of cellular hypertrophy was generally seen among *in vivo* products, particularly within passage 1 T1 products. This observation may be related to the fact that mechanical load as well as biochemical influences can initiate chondroprogenitor cell determination and differentiation but also regulate later stages of chondrocyte maturation and terminal differentiation to the hypertrophic phenotype [33,34].

Finally, Moretti in 2005 demonstrated using articular human chondrocytes within a hyaluronic acid-associated matrix that higher cell density generated more cartilaginous tissue than low cell density. Furthermore it was shown that preculture of the constructs in differentiating medium, but not in proliferating medium, supported enhanced *in vivo* development of engineered cartilage and that the effect of preculture was more pronounced when constructs were seeded at low density as compared with high density [35]. In regard to our results, the parameter preculture or matrix-holding time had strongest outcome effects. Matrix-associated chondrocyte transplantation is solicited by the properties matrix-associated chondrogenic differentiation, even cellular distribution, higher initial stability or better handling [36]. It has been shown by Darling and Athanasiou that goat articular chondrocyte dedifferentiation, which occurred as early as first passage during monolayer proliferation, showed to persist even when the expanded cells were encapsulated within a 3D environment [37]. In another study, human articular chondrocytes were cultured with passage 3 for 1, 2 and 4 weeks on different matrices *in vitro*. It was discovered that mechanical properties of the matrix systems were not improved significantly, a loss of wet weight could be observed and chondrocytes were proliferating only in the early part of cultivation. In addition, the gene expression of collagen type-II was low regarding all matrix systems, while there was a general trend in display of a gradual decline in that expression among the membranes over time [14]. It was furthermore shown [38] that chondrocytes, which have been initially evenly distributed throughout a 3-D membrane, tend to migrate toward the matrix border, which has been termed "edging effect" This effect is in clear conflict with a targeted even distribution, which is likely to be lost over time during *in vitro* culture.

These previous measurements compare very well with the *in vitro* data from our study where all targets measured were inferior after 2 weeks of static *in vitro* culture when compared to the short membrane-holding time. Interestingly, at passage 3, this trend turned around in favor of T2 times for the main differentiation targets when measuring the *in vivo* data. This

phenomenon was solely clearly visible using passage 3 chondrocytes. However, it is very similar to data presented by Lee et al. who in extension touched the idea of preculturing (comparable to T2 in our study). The study group concluded, using a canine ACI model, that a period of 4 weeks of preculturing of passage 3 canine articular chondrocytes, versus 12 h, is beneficial when histomorphometry and certain mechanical properties are concerned. Longer *in vitro* culture resulted in a defect fill with hyaline-like cartilage accounting for 51% of the defect area compared to only 1% in the short-time culture period [39]. We appoint that there is a clear difference between passage 1 and passage 3 chondrocytes when related to varying membrane-holding times. We hypothesize that P1 chondrocytes are phenotypically more stable as well as biosynthetically more active when compared to P3 cells. In that account, passage 1 cells outperform passage 3 cells independent of remaining circumstances and surroundings being occupied by optimal integration and differentiation properties. Per contra, when transplantation is decelerated by *in vitro* membrane-holding times our *in vivo* data demonstrated superior differentiation among P3 cells when compared to P1. Under these conditions the factor precultivation, as described above, may come into play. Phenotypically as well as biosynthetically inferior, the cells to be transplanted may benefit of a certain matrix having been accumulated to protect further integration and differentiation processes since a consolidating matrix has been described to facilitate the cellular response to mechanical load [40]. P1 cells may be able to compensate instability and lack of surrounding matrix when compared to P3 cells. This idea is clearly underlined by a separate investigation of Lee and coworkers who realized that the biosynthetic and proliferative activity of chondrocytes cultured in a similar scaffold (to that used in Ref. [39]) had decreased by four weeks of *in vitro* culture when compared to the short-time culture period [41]. There was no significant difference among passage 5 cells comparing *in vitro* and *in vivo* data, which is most likely related to an advanced dedifferentiation, which the cells were not able to rebound from as previously described by Dell'Accio [27].

Several limitations pertain to this study. A potential transfer of our results to clinical circumstances is limited and large animals may more closely resemble the human. However, it was not practical to conduct initial experiments in larger animals which can be regarded as being in display of current practice [42]. Animals were principally allowed full weight-bearing initially after cessation of anesthesia, which may harm the transplant. However, this setting was similar in all animals remaining circumstances comparable. Furthermore, our press-fit fibrin sealed surgical technique provided with a very secure fixation of the membranes with no signs of delamination during passive free range of motion maneuvers intraoperatively. We chose to use a membrane that is in clinical use, while being aware of the fact that matrix properties can affect cellular behavior [43].

5. Conclusions

An articular chondrocyte expression profile is dependent of and can be enhanced by an optimal orchestration of the cell culture parameters passage, yield and time. Those remain the basic parameters during chondrocyte cultivation. Here, optimal expression patterns concerning chondrogenic differentiation were achieved by few passages, medium cellular yield, short membrane-holding time. Adjusted cell culture parameters may be implemented within clinical ACI to potentially improve transplant quality, long-term durability and therefore patient satisfaction in the future. Large animal studies have to confirm these premature data.

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