Osteoblast-osteoclast co-cultures: a systematic review and map of available literature

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Abstract

Drug research with animal models is expensive, time-consuming and translation to clinical trials is often poor, resulting in a desire to replace, reduce, and refine the use of animal models. One approach to replace and reduce the use of animal models in research is using *in vitro* cell-culture models.

To study bone physiology, bone diseases and drugs, many studies have been published using osteoblast-osteoclast co-cultures. The use of osteoblast-osteoclast co-cultures is usually not clearly mentioned in the title and abstract, making it difficult to identify these studies without a systematic search and thorough review. As a result, researchers are all developing their own methods from the ground up, leading to conceptually similar studies with many methodological differences and, as a direct consequence, incomparable results.

The aim of this study was to systematically review existing osteoblast-osteoclast co-culture studies published up to 6 January 2020, and to give an overview of their methods, predetermined outcome measures (formation and resorption, and ALP and TRAP quantification as surrogate markers for formation and resorption, respectively), and other useful parameters for analysis. Information regarding these outcome measures was extracted and collected in a database, and each study was further evaluated on whether both the osteoblasts and osteoclasts were analyzed using relevant outcome measures. From these studies, additional details on methods, cells and culture conditions were extracted into a second database to allow searching on more characteristics.

The two databases presented in this publication provide an unprecedented amount of information on cells, culture conditions and analytical techniques for using and studying osteoblast-osteoclast co-cultures. They allow researchers to identify publications relevant to their specific needs and allow easy validation and comparison with existing literature. Finally, we provide the information and tools necessary for others to use, manipulate and expand the databases for their needs.

Introduction

Bone is a highly dynamic tissue with mechanical and metabolic functions that are maintained by the process of bone remodeling by the bone forming osteoblasts (OBs), bone resorbing osteoclasts (OCs), and regulating osteocytes. In healthy tissue, bone resorption and formation are in equilibrium, maintaining the necessary bone strength and structure to meet the needs of the body. In diseases such as osteoporosis and osteopetrosis this equilibrium is disturbed, leading to pathological changes in bone mass that adversely affect the bone's mechanical functionality (1).

Studies on bone physiology, bone disease and drug development are routinely performed in animal models, which are considered a fundamental part of preclinical research. The use of animals raises ethical concerns and is generally more time consuming and more expensive than *in vitro* research. Laboratory animals are also physiologically different from humans and their use in pre-clinical studies leads to poor translation of results to human clinical trials (2,3), and the subsequent failure of promising discoveries to enter routine clinical use (4,5). These limitations and the desire to reduce, refine and replace animal experiments gave rise to the development of *in vitro* models (6,7). Over the last four decades, significant incremental progress has been made towards developing OB-OC co-culture models.

The development of *in vitro* OB-OC co-cultures started with a publication of T.J. Chambers in 1982 (8), where the author induced quiescence of isolated tartrate resistant acid phosphatase (TRAP)-positive rat OCs with calcitonin and reversed their quiescence by co-culturing them with isolated rat OBs in direct contact. At that time, studies involving OCs resorted to the isolation of mature OCs by disaggregation from fragmented animal bones. The first account of *in vitro* osteoclastogenesis in co-culture was realized in 1988 when Takahashi and co-authors (9) cultured mouse spleen cells and isolated mouse OBs in the presence of 1α,25-dihydroxyvitamin D3 and found TRAP-positive dentine-resorbing cells. The herein described methods were used and adapted to generate OCs for the

following decade. Most of the studies published until this point in time used co-cultures as a tool for achieving osteoclastogenesis, as opposed to a model for bone remodeling. At that time, a co-culture of OBs with spleen cells or monocytes was the only way of generating functional OCs *in vitro*. It wasn't until 1999 that Suda (10) discovered Receptor Activator of Nuclear Factor Kappa Ligand (RANKL) and Macrophage Colony Stimulating Factor (M-CSF) as the necessary and sufficient proteins required for differentiating cells from the monocyte/macrophage lineage into functioning OCs (11–13). This discovery marked the start of co-culture models developed for studying bone remodeling.

In recent years, many research groups have ventured into the realm of OB-OC co-cultures with the intent of studying both formation and resorption, but each group seems to be individually developing the tools to suit their needs resulting in many functionally related experiments that are methodologically completely different. In addition, the use of such methods is often not clearly stated within title and abstracts. Simple title/abstract searches such as 'OB + OC +co-culture' tend to scratch only the surface of the base of evidence available using OB-OC co-cultures. Finding and comparing different co-culture approaches and results is thus virtually impossible and forces each group to develop and use their own methods instead of building upon those of others.

The aim of this study was to construct a systematic review of all OB-OC co-cultures published up to January 6, 2020. With this systematic review, we aimed at identifying all existing OB-OC co-culture studies and analyze these within two comprehensive databases, allowing researchers to quickly search, sort and select studies relevant for their own research. Database 1 contains all OB-OC co-culture studies in which at least one relevant primary outcome measure was investigated (formation and/or resorption) or secondary outcome measure (alkaline phosphatase (ALP) and/or tartrate resistant acid phosphatase (TRAP) quantification as surrogate markers for formation and resorption, respectively) (S1_File_Database_1). A sub-selection of studies that investigated these relevant outcome measures on both OBs and OCs in the co-culture was included in Database 2, accompanied

by additional details on methods, culture conditions and cells (S2_File_Database_2). The collection of the two databases will further be referred to as a systematic map.

Methods

For this systematic map a structured search protocol was developed using the SYRCLE protocol format (14). This protocol format is tailored to the preparation, registration, and publication of systematic reviews of preclinical studies, and helps authors predefine the methodological approach of their review from research question to data synthesis. The protocol and search strings were made publicly available before completion of the study selection via Zenodo (15) to ensure transparency of the publication. In short, three online bibliographic literature sources were consulted with a comprehensive search string and the resulting publications were combined and screened using a four-step procedure (Fig. 1): 1) identification of OB-OC co-cultures, 2) identification of relevant outcome measures, 3) categorization in Databases 1 and 2 (Fig 2), 4) search for additional articles in the reference lists of studies included in Database 2 and relevant reviews.

Fig 1. Flow diagram of systematic literature search and screening*. Screening step 1: Hits from 3 online bibliographic literature sources were combined, primary studies were selected, and duplicates were removed. Title and abstracts were screened for the presence of OB-OC co-cultures. Screening step 2: OB-OC co-cultures were screened in full text for relevant outcome measures. All studies in which at least one relevant outcome measure was studied were included into Database 1. Screening step 3: Papers in which both cell types were studied with relevant outcome measures were included into Database 2. Screening step 4: Papers included into Database 2 and relevant reviews were screened for potentially missing relevant studies and identified studies were screened in the same manner as described here. Each screening step is marked with a separate background color. Each selection step within the screening steps is marked with a colored header. Blue header: used as input for the review.*

Grey header: selection step. Red header: excluded studies. Yellow header: Database as presented in this systematic map. Abbreviations: outcome measures (OM), Database 2 (DB2), osteoblast (OB), osteoclast (OC).

***Fig 2. Schematic overview of Databases 1 and 2**. All identified studies were searched for OB-OC co-cultures, where co-culture was defined as OB and OC being present simultaneously and able to exchange biochemical signals. In addition to direct-contact cultures, cultures such as transwell cultures, 3D or scaffold cultures and bioreactor cultures were allowed as well. OB-OC co-culture studies which used relevant outcome measures were included into Database 1. Of these, only the relevant outcome measures were analyzed. All studies where relevant outcome measures were used for both OB and OC were included into Database 2 as well. Of these, cells and culture conditions were analyzed. The figure was modified from Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License (http://smart.servier.com, accessed on 2 July 2021).*

Database Search

The online bibliographic literature sources Pubmed, Embase (via OvidSP) and Web of Science were searched on January 6, 2020 with a predefined search query developed to identify as many studies as possible employing OB-OC co-cultures. The search strings used a combination of thesaurus and free text terms where possible and consisted of the following components: ([OBs] OR ([OB precursors] AND [bone-related terms])) AND ([OCs] OR ([OC precursors] AND [bone-related terms])) AND [co-culture], where each component in square brackets represents a list of related thesaurus and free-text search terms, and where parentheses indicate the order of operations within the search query. The full search strings can be found via Zenodo (15). The results of all three searches were combined. Conference

abstracts and duplicates were removed using the duplicate removal tools of Endnote X7 and Rayyan web-based systematic review software (16).

The remaining entries were screened using a four-step procedure that resulted in the generation of 2 databases: Database 1 containing all co-culture studies that measured at least one relevant outcome measure (formation, resorption, ALP or TRAP), and Database 2 containing all studies that measured at least one relevant outcome measure on both OB and OC: either formation or ALP for OB, and either resorption or TRAP for OC. The entire data collection and screening process was performed independently by two researchers starting from the moment that conference abstracts and duplicates had been removed and ending the moment that the relevant information of the publications was extracted into the two databases. Disagreements between researchers were discussed and publications were re-screened if necessary. A third independent researcher was consulted for disagreements that could not be solved by the first two independent researchers.

Screening step 1: Identification of OB-OC co-cultures

This step was performed to identify and extract OB-OC co-cultures from the complete list of studies identified from the three online bibliographic literature sources after automatic removal of conference abstracts and duplicates. All further steps were done on these studies or a sub-selection thereof. Using Rayyan web-based systematic review software (16), the titles and abstracts of all entries were screened for the presence of primary studies using OB-OC co-cultures. Reviews, theses, chapters, and conference abstracts that were not automatically detected were excluded at this point. Potentially relevant reviews were saved separately to serve as an additional source of studies that could have been missed by the systematic search.

In the selection process, co-culture was defined as the simultaneous presence (verified) or assumed presence (expected) of OBs and OCs (or OB-like and/or OC-like cells) within the same culture system

at a moment during the described experiment such that the cells were able to communicate either via soluble factors in the medium and/or direct cell-cell contact. Both primary cells and cell lines of any origin were admitted including heterogeneous cell populations, if these were clearly defined and expected to result in a biologically relevant number of the desired cell type, precursor type, or terminally differentiated cell type. The presence of progenitor cells (such as monocytes or mesenchymal stem/stromal cells) was allowed only if these were either verified or expected to differentiate into OBs and/or OCs. Studies using a single animal or human donor for both cell types were allowed, but only if the two (progenitor) cell types were at one point separated, counted, and reintroduced in a controlled manner. In addition, trans-well systems (no physical contact but shared medium compartment with or without membrane), scaffolds (3-dimensional porous structure of any material including decellularized matrix), and bioreactor culture systems (culture exposed to physical stimuli such as rotation, mechanical loading or fluid flow) were included. Conditioned media experiments were excluded because these do not allow real-time two-way exchange of cell signals. Explant cultures or organ cultures were excluded because these studies contain a living *ex vivo* culture element, whereas the focus of this systematic map is limited to *in vitro* studies.

When the study used any type of OB-OC co-culture as defined above, the study was included. When there was no indication that there was an OB-OC co-culture, the study was excluded. When, based on the title and abstract, it was likely that there was a co-culture, but this was not described as such, the full-text publication was screened.

Screening step 2: Identification of relevant outcome measures in the co-culture experiments

This step was used to identify co-cultures that specifically investigated outcome measures related to bone remodeling: measuring formation or resorption (primary outcome measures), or quantitative

184 measurements of activity markers ALP or TRAP in a dedicated assay (secondary outcome measures).
185 The primary outcome measures of measuring resorption and formation were chosen because these
186 are the processes that are directly affected in bone diseases. Measuring these outcome measures
187 usually requires a specific methodological setup such as a specific surface analysis for measuring
188 formation, or a resorbable substrate for measuring resorption. The secondary outcome measures of
189 ALP and TRAP were included because these are regarded as viable alternatives for the direct
190 measurement of formation and resorption. The full texts of the studies identified in screening step 1
191 were screened for experimental techniques and outcome measures. Studies in which for at least one
192 of the cell types a relevant outcome measure was used, were selected to be used in Database 1
193 (S1_File_Database_1). The measurement of formation was defined as any method that directly
194 measures the area or volume of (tissue) mineralization by OBs, any method that measures by-products
195 of formation, and any method that measures biochemical markers that directly and exclusively
196 correlate to formation. The measurement of resorption was defined as any method that directly
197 measures the surface area or volume that has been resorbed by OCs, that biochemically measures
198 products or by-products of resorption, or that measures biochemical markers that directly and
199 exclusively correlate to resorption. The measurement of ALP and TRAP was defined as the detection
200 of either the direct measurement of the enzymatic activity of these proteins, or the direct
201 quantification of the amount of those proteins present in a dedicated assay. Studies that determined
202 ALP or TRAP gene expression using PCR were excluded because PCR was not considered a dedicated
203 assay for this map and did not directly measure the amount of protein present. However, the use of
204 PCR was recorded in the generated databases in a separate column. Immuno-histological stainings of
205 ALP or TRAP were not considered relevant outcome measures, even when followed by image analysis
206 because at best these quantify stained surface area and not actual protein content.

207 All co-cultures that did not contain at least a single outcome measure that met these criteria were
208 excluded from further use. Because this was the first step at which the availability of the full text

publication was required, publications written in languages other than English with no translation available, and publications of which the full text could not be found were excluded at this point.

Screening step 3: Categorization within Database 1

In this step, a distinction was made between studies in which only one of either OB or OC was studied, or both were studied. This distinction was made because ideally, a model for bone remodeling should show effects on both OBs and OCs. Each study selected from screening step 2 was assessed on the methods to study OBs and OCs. Each study was categorized into one of five categories within Database 1: 1) The relevant outcome measures were measured in both OBs and OCs in the co-culture. These studies were also included in the in-depth screening for Database 2 (S2_File_Database_2). 2) Both cell types were studied, but relevant outcome measures were only measured in OCs or 3) Both cell types were studied, but relevant outcome measures were only measured in OBs. 4) Only OCs were studied in co-culture, the other cell type was neglected or 5) OBs were studied in co-culture, the other cell type was neglected. Thus, category 1 contained the studies in which both formation and resorption were investigated, either directly or by ALP or TRAP quantification. Category 2 and 3 contained studies in which both OBs and OCs were studied, but only one of the two was studied with the relevant outcome measures. The other cell type was studied using other methods instead such as stainings or PCR. Categories 4 and 5 contain studies in which only one of the two cell types was analyzed with one of the relevant outcome measures while the other cell type was present but not analyzed in co-culture at all. Note that for this categorization, it was necessary that the cells that were used in co-culture were studied, and not for example a mono-culture conducted in parallel.

Screening step 4: Review and reference list screening

To find additional studies that may have been missed during bibliographic searches, relevant review articles identified during the selection process and studies labeled as category 1 in step 3 were screened for additional publications that could be relevant to the current systematic map. Of these studies, the relevant passages within the text were screened, followed by a thorough screening of the complete reference lists of these studies. All potentially relevant studies were first cross-checked with the original search results of the bibliographic literature search, and if these were not identified there, were screened in the same manner as all other studies used in this systematic map. Unique relevant studies were then added to the corresponding databases and analyzed as described earlier.

Database 1 generation and analysis – All co-cultures with relevant outcome measures

Every study included in Database 1 was screened for the relevant outcome measures resorption, formation, ALP and TRAP during screening steps 2 and 3. To provide useful and specific information of each of the studies included in this database, all potentially relevant information related to the relevant outcome measures was collected and organized. For resorption, additional information on the resorbed substrate, the methodological procedure and quantification of results was collected. For formation, additional information on the type of analysis, the methodological procedure and quantification of results was collected. For both ALP and TRAP, additional information on the mechanism of the biochemical assay, whether it was conducted on lysed cells or supernatant, and information regarding the quantification was collected. In addition, the following information was extracted, whether: the authors described their setup as a model specifically for remodeling, the experiment was conducted in 3D, the experiment applied bioreactors, more than 2 cell types were cultured simultaneously, the culture used a trans-well setup, the culture used PCR and components in the supernatant of the culture were analyzed by ELISA or a similar quantification method. If the answer

to these questions was yes, then the applicable details were collected as well. Finally, a column for additional remarks was introduced for details that did not fit in another column. Studies where the authors are color coded in pink were those not found through the initial database search but by the screening of the review articles or reference lists. Studies categorized as category 1 in screening step 3 were selected for use in Database 2 and had their title color coded in orange.

Quality assessment and scripting

In Database 1 only the methods used for analyzing relevant outcome measured are reported, and not the data obtained from them or the results described in the publication. Quality assessment in Database 1 is thus limited to assessing the completeness of the necessary elements of the collected methodological details, to the extent that the methods are properly represented in Database 1 and related tables. Please note that the methods themselves were not investigated on a complete description for a perfect replication of the study, but only on the description necessary to accurately classify the method within this systematic map. For example, a study claiming to investigate resorption on dentine discs using Toluidine Blue was deemed sufficiently described to accurately classify, regardless of whether the information presented was sufficient to duplicate that specific method precisely. Publications in which information was missing are here represented as 'not reported' if no information was provided, 'reference only' if no information was provided but another study was referenced, and 'undefined kit', when a commercial kit was used but the content or methodology was not further described. Instances of missing information can easily be identified in figures, tables and databases, but were not further used in this systematic map. Studies where an instance of information was missing were still used for other analyses for which the corresponding provided information was present.

A script was written in Excel Visual Basics programming language to analyze Database 1 and extract relevant statistical information on the collected information. On sheet 2 "Data" of the Database 1 excel file, the descriptive statistical data and collected information are presented in the form of lists

and tables and together with a button to re-run the analysis based on the reader's requirements. The script is integrated within the excel file and can be used only when the file is saved as a 'macro-enabled' file (.xlsm).

Database 2 generation and analysis – All co-cultures in which both cell types had relevant outcome measures.

In addition to the information already collected for Database 1, additional information was extracted from the studies in which relevant outcome measures were studied of both OB and OC: the species (17) and type (cell line or primary) and actual used cell type (6) of both the OB and OC were collected. Seeding numbers and densities (18) for both OB and OC were collected or calculated where possible, separated by 2D (cell density per area) and 3D (cell density per volume), and the seeding ratio (19) between OB and OC was noted or calculated. The culture surface (bio-)material (20), sample size (samples per group), culture duration and medium refreshing rate in units as reported in the study, environmental conditions or variations such as CO_2 or O_2 alterations or mechanical loading (21), and pre-culture duration (22) were collected, where pre-culture is defined as a different co-culture condition (such as a different supplement cocktail) lasting for a short duration (such as 2 days) prior to the 'main' co-culture. The medium composition (23) was collected and organized by base medium type such as Dulbecco's Modified Eagle Medium (DMEM) and alpha-Modified Eagle Medium (αMEM), glucose content (if provided separately in the text), Fetal Bovine Serum (FBS) / Fetal Calf Serum (FCS) in percentages, antibiotics (types and concentrations or percentages as provided), OB supplement concentrations (ascorbic acid, β-glycerophosphate and dexamethasone, OC supplement concentrations (M-CSF and RANKL) and other supplements, as well as medium content of any monoculture prior to the co-culture. Finally, the tested genes of all studies applying PCR and any proteins studied with ELISA or other supernatant analyses executed on the co-culture were noted.

Quality assessment and scripting

In Database 2 the culture conditions, cells and materials used are reported, and not the data obtained from them or the results described in the publication. Quality assessment in Database 2 is thus limited to assessing the completeness of the necessary elements of the collected methodological details, to the extent that the methods are properly represented in Database 2 and related figures and tables. Please note that the methods themselves were not investigated on a complete description for a perfect replication of the study, but only on the description necessary to accurately classify the method within this systematic map. For example, a study claiming to use human primary monocytes and human primary osteoblasts for the OB-OC co-culture was deemed sufficiently described to accurately classify respectively the OB and OC origin, regardless of whether the information presented was sufficient to perfectly replicate that part of the study. Publications in which information was missing are here represented as 'not reported' (NR) if no information was provided, or 'reference only' if no information was provided but another study was referenced. If studies were missing information critical to reproduce the outcome measures (for example seeding ratio's, culture surface material, medium or supplement information, critical steps in analyses), the cells in the database missing this information were labeled in red. If the missing information was not critical for the outcome measures but necessary for a replication of the study (for example sample size, medium refresh rate, control conditions), the cells were labeled in orange. The sum of both orange and red cells for each color in each study is shown as well to indicate how many instances of missing information were identified in each study. The color coding was determined by the authors of this map but can be adjusted within Database 2 if other criteria for critical information and completeness are desired. Instances of missing information can easily be identified in the corresponding figures, tables and databases, but were not further used in this systematic map. Studies where an instance of information was missing were still used for other analyses for which the corresponding provided information was present.

Using Excel Visual Basics programming language, three scripts were written to analyze and process Database 2. One script was created to count all instances of cells labeled as 'missing info' and present this number in two dedicated columns (missing critical or non-critical info). One script was created to

count the frequency of occurrence of all (co-)authors and years of publication. Finally, one script was created to analyze this database and extract relevant descriptive statistical data on the collected information. On sheet 2 “Data” of the Database 2 excel file, the statistical data and collected information are presented in the form of lists and tables and together with the buttons to re-run the analyses based on the reader's requirements. The scripts are integrated within the excel file and can be used only when the file is saved as a ‘macro-enabled’ file (.xlsm).

Results

Search results

From three online bibliographic literature sources, 7687 studies were identified (Pubmed: 1964, Embase via OvidSP: 2709, Web of Science: 3014). After removal of conference abstracts, 6874 studies remained. After duplicate removal, 3925 unique studies were identified to be screened.

Studies included into Database 1

After title-abstract screening and when in doubt full text screening (screening step 1), 694 studies were identified as OB-OC co-cultures. A list of these studies is available as a supplementary file (S4_File_List of all OB-OC co-cultures). Of these, one study was excluded from further analysis because the full text could not be obtained, 35 were excluded because they were in a language other than English and 406 were excluded because no relevant outcome measure was used in the study (screening step 2). The qualifying 252 studies with at least one relevant outcome measure were included in Database 1.

Studies included into Database 2

In 77 of these studies, both the OB and OC were studied, and in 39 of these, both OB and OC were studied using relevant outcome measures (screening step 3). These 39 studies were included in Database 2.

Additional screening of review articles and reference lists for missing studies

The 39 studies of Database 2 and 10 additional review publications were screened for other relevant studies that the initial search may have missed (screening step 4). An additional 25 unique studies were identified in the 10 reviews, and 34 unique studies were identified from the reference lists of the included studies. These additional 59 studies were reviewed as described previously and resulted in an additional 3 OB-OC co-cultures with only relevant outcome measures measured on one cell type, resulting in a total of 255 studies with relevant outcome measures on at least one cell type for Database 1, and 39 studies in which relevant outcome measures were studied in both cell types for Database 2. A detailed overview of the search and selection process is shown in Fig 1.

Publications per year

The publications included in Database 1 were published between 1983 and 2019, with a peak in publications around the year 2000, followed by a dip and then a more or less slight but steady increase until now (Fig 3a). The peak roughly coincides with the discovery that M-CSF and RANKL were both necessary and sufficient to induce osteoclastic differentiation in monocytes in 1999 (10). The included publications in Database 2 span the time between 1997 and 2019, with only 8 publications before 2010 (Fig 3b). This coincides with the progress in development of *in vitro* co-cultures of OB and OC,

375 moving beyond co-cultures with OB to generate OC, and moving towards co-cultures of OB and OC to
376 study for example cell-cell interactions (6).

377

378 ***Fig 3. Relevant publications per year.*** *A) All 255 publications that contain relevant outcome measures*
379 *counted by year ranging from 1983 to 2019 (Database 1). B) The 39 selected publications of Database*
380 *2 counted by year ranging from 1998 to 2019 (Database 2).*

381

382 Database 1 results

383 Database 1 provides an overview of all OB-OC co-culture studies published until January 6, 2020 in
384 which at least one relevant outcome measure was studied. Of the 255 studies included, resorption
385 was analyzed in 181 studies, formation was analyzed in 37 studies and both were analyzed in 16
386 studies. ALP was analyzed in 42 studies, TRAP was analyzed in 61 studies and both were analyzed in
387 22 studies (Table 1).

388 **Table 1. Combinations and frequencies of primary and secondary outcome measures.**

Combinations of primary and secondary outcome measures in each study		Primary outcome measures				
		No resorption or formation	Resorption only	Formation only	Resorption and formation	Total
Secondary outcomes measures	No ALP or TRAP	0	151	14	9	174
	ALP only	16	0	2	2	20
	TRAP only	23	9	3	4	39
	ALP + TRAP	14	5	2	1	22
	Total	53	165	21	16	255

Table 1: *This table can be referenced to identify the number of studies using any combination of primary and secondary outcome measures. All 255 studies that investigate at least one of the primary or secondary outcome measures are represented once in this table. Each study is represented by a combination of primary outcome measures (horizontal) and secondary outcome measures (vertical). Marginal totals of each row and column are counted under 'total' with the grand total in the bottom-right cell. These marginal totals sum the total number of studies that studied only that combination of either primary or secondary outcome measures, with no regard of the outcome measures on the opposite axis. The total numbers of each individual outcome measure can be calculated from this table but are presented in the following paragraphs and tables.*

Resorption

Resorption is the process by which osteoclasts remove old and damaged bone tissue through enzymatic degradation or acidic dissolution. Out of all 255 OB-OC co-culture publications included in Database 1, resorption was studied directly on 188 occasions in 181 studies and quantified 142 times (Table 2). In some publications, more than one material or method of analysis for resorption was used. In cases where multiple materials were used, each material was counted as an individual study of resorption and for each material, the corresponding analyses were counted, even if these were identical per material. In those cases where multiple methods of analysis are used on the same material, all methods are counted, and the material is counted only once. This resulted in a counted number of studies that is higher than the actual number of publications. When numbers of studies are referenced, these are the 'counted' number of studies defined above, and not the actual number of publications.

Table 2a. Occurrences of resorption on different types of substrates and subsequent analyses.

		Materials used as a resorbable substrate for measuring resorption											
Shapes, structures and types of materials used as resorbable substrate for analysis of resorption.		**Dentine**	**Bone**	**HA**	**Silk**	**Collagen**	**CaP**	**PLLA**	**Chitosan**	**Osteologic**	**Mineralized**	**Not reported**	**Per-row total**
Per-material total number of studies		76	66	6	5	2	4	1	1	19	6	2	188
Per material quantified studies		55	52	3	4	1	4	0	0	17	4	2	142
Shape or structure of material	**Discs**	76	63	2			2			13			156
	Films			2	4			1	1				8
	Coatings			2			1						3
	Scaffolds				1	1					3		5
	Hydrogels					1							1
	ECM										2		2
	Nodule										1		1
	Fragments		3										3
	Substrates						1						1
	Plates									6			6
	Not reported											2	2
Analysis techniques for analzying resorption on resorbable substrates.		**Dentine**	**Bone**	**HA**	**Silk**	**Collagen**	**CaP**	**PLLA**	**Chitosan**	**Osteologic**	**Mineralized**	**Not reported**	**Per-row total**
Staining	**Toluidine Blue**	36	19										55
	Haematoxylin	16	2										18
	Eosin		1										1
	H&E		1										1
	Alum / Coomassie Blue		1										1
	TRAP	1											1
	Von Kossa						2			4	1		7
Microscopy	**Phase contrast**						1			4			5
	SEM	12	37	5	3		1			1			59
	TEM					1					1		2
	2-Photon										1		1
	Atomic force				1			1	1				3
	Reflected light	8	2										10
	Dark field									1			1

	Light microscopy									6			6
Other	**Assay**					1							1
	Immuno-assay		3								3	1	7
	MicroCT				1					1			2
	Reference only	2											2
	Not reported	1		1						2		1	5
	Total per material	76	66	6	5	2	4	1	1	19	6	2	188

Table 2a: *Each column signifies a different material used as a substrate for measuring resorption. If other cells, prior to the introduction of OC, were used to deposit mineralized matrix on another material, then the material was listed in the column 'Mineralized'. If the material was not reported, the study was listed in the column 'Not reported'. The first rows show how many instances of each material were included into this systematic map in total, and how many times the results were quantified. The final column shows incremental totals per material type or analysis type. This table consists of two sections. The top section shows in what form or shape the corresponding materials were used as a substrate for resorption. The bottom section shows the techniques that were used to study the resorption described on the materials described in the top section. Each individual study is represented exactly once in the top section of the table to signify the type and form of the substrate used, and exactly once in the bottom section of the table to signify the method used to analyze the resorption that occurred on that substrate. This required the selection of the most 'important' part of the methods used. In the cases where first a staining was used followed by microscopy, only the staining is listed. Only in those cases where resorption was investigated directly with a microscope without prior staining, the type of microscopy is listed.*

Table 2b Supernatant resorption techniques

Supernatant Analysis techniques per material used for analysis of resorption.	**dentine**	**bone**	**HA**	**silk**	**collagen**	**CaP**	**PLLA**	**chitosan**	**osteologic**	**mineralized**	**not reported**	**Per-row total**

Supernatant	**NTx**	1									2		3
	CTx										1	1	2
	ICTP		1										1
	Phosphate release					1					2		3
	Radioactive proline release		2										2

Table 2b: *This table presents five types of resorption analysis where measurements can be performed in the culture supernatant and not on the material itself. In the corresponding studies, these were done in addition to 'regular' analysis presented in Table 2a, and for that reason are presented separately. These have the advantage that they can be used to monitor changes over time in a non-destructive way.*

Most of these studies used discs or fragments of either bone or dentine. Due to the flat nature of these discs, the surface can be considered 2D, and resorption pits can be visualized directly using conventional microscopy techniques, such as for example Scanning Electron Microscopy (SEM) or Reflected Light Microscopy (RLM). To enhance the contrast of the resorption pits, stains such as Toluidine Blue (TB) and Hematoxylin (H) were used. Resorption on bone fragments was quantified using radioimmunological assays measuring the release of *in vivo* pre-labeled ^{3}H-proline or type I collagen telopeptide.

Synthetic resorbable discs or coatings on culture plates are designed specifically for studying resorption, and usually the exact composition has not been revealed. These will further be referred to as 'osteologic' plates or discs. The discs were analyzed in roughly the same way as bone or dentine discs. Thin resorbable coatings on translucent culture plates offer another interesting approach. Resorbed areas reveal the translucent culture plate, while unresorbed areas are less translucent and can be stained with for example von Kossa's method to provide even more contrast, making quantification with image analysis easy.

Hydroxyapatite (HA) and other calcium phosphates were used in the form of discs, films, coatings, or scaffolds and were analyzed using various types of microscopy, both with and without prior staining.

These can be used in a similar way as biological and synthetic materials mentioned earlier, with the main advantage being their known composition.

Resorption of ECM or nodules produced by OBs and scaffolds mineralized by OBs were investigated with transmission electron microscopy, light microscopy after staining, using 2-photon Second Harmonic Generation microscopy (24), supernatant phosphate levels, or with an ELISA for C-terminal telopeptide (CTx) or N-terminal telopeptide (NTx), which are bone turnover marker more commonly used for testing urine and serum samples.

Formation

Formation is the process by which osteoblasts create new bone tissue through the mineralization of deposited collagenous extracellular matrix. Out of all OB-OC co-cultures included in Database 1, formation was studied directly 39 times in 37 studies and quantified 29 times. (Table 3) In some studies, more than one method of measuring, analyzing and quantifying formation was used. In those cases, all methods are counted as individual studies. The methods of formation analysis were divided into 5 types: nodule analysis, volume analysis, surface analysis, supernatant analysis and 3D scans.

Table 3: Formation statistics and analyses.

		Type of analysis used to measure formation					
	Technique	**Scan**	**Nodule analysis**	**Supernatant analysis**	**Surface analysis**	**Volume analysis**	**Per-row Total**
	Total	3	20	6	5	5	39
	Quantified	3	12	6	3	5	29
Me	**Scaffold**	2	1	1	3	2	9

asu red	**Film**	1		1	1	2	5
	Hydrogel					1	1
	Pellet		1			1	2
	Dye release		5				5
	Analysis	Scan	Nodule analysis	Supernatant	Surface analysis	Volume analysis	Per-row Total
Staining	**H&E**				1		1
	Von Kossa		2		1		3
	Alizarin Red		16				16
	Lentiviral fluorescence		1				1
Assays	**Calcium**					3	3
	Calcium + Phosphate					2	2
	CICP			6			6
Other	**SEM**		1		3		4
	microCT	3					3
Per-analysis Total		3	20	6	5	5	39

Table 3: *Each column signifies a different type of analysis used for measuring formation. These include any type of non-destructive scan, a form of analysis of mineralized nodules, supernatant analysis, surface analysis, and destructive analysis of a mineralized volume. The first rows show how many instances of each type of analysis were included into this systematic map in total, and how many times the results were quantified. The final column shows marginal totals per row of each row. This table consists of two distinct sections, each starting with a row showing all analysis types for convenience. The first section lists defining characteristics of studies such as using films, scaffolds, hydrogels or*

pellets, or using a technique to first stain tissue, and then releasing and measuring the released dye. Not each study had such defining characteristics, and the total of section one does not add up to 39 studies. Section two shows either which materials was measured, or which technique was used for measuring formation. Each instance of formation is represented in section two of this table exactly once. Stainings were followed by microscopy or an assay in those cases where dye was released to be measured.

The most common method to quantify formation was to investigate mineralized nodule formation. This was done by using staining techniques such as Alizarin Red (calcium) (25) or von Kossa (phosphate) (26) followed by imaging, or directly imaging the nodules. While any staining specific for mineralized matrix or even plain light microscopy images could be quantified using appropriate imaging techniques and software, Alizarin Red offers an additional way of quantification: the amount of dye binding to the mineral correlates to the amount of mineralization, after imaging the dye can be released from the minerals using acetic acid and can then be quantified using colorimetric spectrophotometry (27). Surface analysis was used in a similar way to study formation on scaffolds, films, or particles. Scaffolds were stained and/or imaged, and the area of matrix deposition was visualized or quantified. Volume analysis was used to describe the measurement of mineralized tissue components calcium and phosphate, which were released after destruction of the matrix. These three types of formation measurement are destructive methods, meaning that the samples must be sacrificed for each time point.

The remaining two types of formation methods are non-destructive. Supernatant analysis was used to describe the measurement of Collagen type I C-terminal propeptide (CICP), a byproduct of collagen

497 deposition, in cell culture supernatant. 3D scan was used to describe the use of (in this case) µCT
498 quantify the three-dimensional structure of mineralized matrix.

499

500 TRAP measurements as a surrogate marker of osteoclastic resorption

501 TRAP is a protein that has long been used as the predominant OC marker (28). Out of all OB-OC studies
502 in Database 1, TRAP was studied 63 times in 61 publications by a dedicated assay (Table 4). TRAP can
503 be measured intracellularly or excreted into the medium in two ways. Its enzymatic phosphatase
504 activity can be measured directly, or the amount of TRAP molecules present can be quantified. TRAP
505 release was studied both on cell lysate and on supernatant, and in some cases on both. The most
506 frequently used method to study TRAP activity was using 4-nithophenylphosphate (pNPP), a substrate
507 that is cleaved by phosphatases into phosphate and detectable yellow 4-nithophenol. Others used the
508 fluorophore Naphthol ASBI-phosphate, which is converted into the fluorescent Naphthol-ASBI (29)
509 and shows specificity for TRAP isoform 5b, making this method more specific for the detection of OC
510 when compared to the measurement of TRAP enzyme in general (30). Naphtol ASMX phosphate (31)
511 and an otherwise undisclosed diazonium salt function in a similar manner. Enzyme linked
512 Immunosorbent Assay (ELISA) can be used to detect TRAP in a slightly different manner; by binding a
513 detectable substrate directly to the TRAP enzyme instead of using the enzyme to produce detectable
514 substrate. Relying on conjugated enzymes or fluorescence, these techniques should be more effective
515 at low concentrations of TRAP because multiple conjugates could bind to a single TRAP molecule.
516 Others used a kit to detect TRAP, but no description of the assay other than the manufacturer were
517 given.

518 **Table 4: TRAP measurement techniques and analyses.**

Type	pNPP	N-ASBI-P	N-ASMX-P	ELISA	Diazonium salt	Undefined kit	Reference	Not reported	Total
Total	33	5	1	9	1	9	4	1	63

Lysed cells	29	5	1	1		3	2		41
Supernatant	6			7	1	6	2		22
Reference only				1					1
Not reported						1		1	2
Analysis	pNPP	N-ASBI-P	N-ASMX-P	ELISA	Diazonium salt	Kit	Reference	Not reported	Total
absorbance	33		1	8		6	2	1	51
Fluorescence		5							5
Radiography									0
Reference only	2			1	1		2		6
Not reported						4			4

519

520 ***Table 4:*** *Each column in Table 4 signifies a different technique to measure TRAP. This table consists of*
521 *two distinct sections. The first section shows the number of studies that used each technique, and*
522 *whether these were used on (lysed) cells or on culture supernatant. If only a reference to other*
523 *literature was provided, that instance was listed in the row 'Reference only', and when these details*
524 *were not reported, that instance was listed in the row 'Not reported'. Note that in a single study TRAP*
525 *can be measured with the same technique on both cell lysate and culture supernatant, resulting in a*
526 *higher count of occurrences than number of studies that analyzed TRAP. The second section shows*
527 *with which method of analysis the TRAP content was measured. If only a reference to other literature*
528 *was provided, that instance was listed in the row 'Reference only', and when these details were not*
529 *reported, that instance was listed in the row 'Not reported'. If one study measured TRAP on both cells*
530 *and supernatant, then that study is represented twice in the second section. In all other cases, each*
531 *study is represented once in each section.*

532

533 ALP measurements as a surrogate marker of osteoblastic tissue formation

534 Alkaline phosphatase (ALP), a bone turnover marker that is commonly used to investigate OBs, was
535 studied in 42 publications (Table 5). ALP is a phosphatase, that like TRAP in OCs can be found both

536 within and on the OBs surface and can be excreted into culture medium soluble or via extracellular
537 vesicles (32,33). The most frequently used method to measure ALP was to use the substrate pNPP,
538 which is cleaved by ALP into phosphate and detectable yellow 4-nitrophenol. Enzyme Immuno Assays
539 (EIA) and ELISAs are similar immunoenzymatic assays (34) that rely on an labelling ALP molecules with
540 a detectable substrate or other enzymes. This is in contrast with the pNPP-based methods, where the
541 ALP enzyme itself through its inherent enzymatic activity is responsible for generating the colored
542 substance. An advantage of the EIA and ELISA methods is that these are generally more sensitive;
543 multiple detectable molecules or enzymes can be bound to each ALP molecule. Others used a kit to
544 measure ALP, but no description of the assay other than the manufacturer were given.

545 **Table 5: ALP measurement techniques and analysis.**

		ALP measurement techniques				
	Type	pNPP	EIA	ELISA	Undefined kit	Total
Substrate	Total	26	8	1	7	42
	Lysed cells	19	1		6	26
	supernatant	8	7	1	2	18
Detection	absorbance	25	8	1	3	37
	Reference only	2				2
	Not reported				5	5

546

547 ***Table 5:*** *Each column signifies a different technique to measure ALP. The first rows show the*
548 *occurrence of each technique and whether these were used on (lysed) cells, or on culture supernatant.*
549 *Note that in a single study ALP can be measured with the same technique on both cell lysate and culture*
550 *supernatant, resulting in a higher count of occurrences than number of studies that analyzed ALP. The*
551 *final three rows show with which method of analysis the ALP content was measured.*

552

553 Database 2 results

554 While Database 1 was created to provide an overview of all reported methods to study the relevant
555 outcome measures (resorption, formation, TRAP and ALP) without other experimental details,
556 Database 2 was created to provide more insight into what culture conditions were used for co-
557 cultures. From Database 1, studies that investigated relevant outcome measures on both OB and OC
558 were regarded as co-cultures capable of showing OB-OC interaction, versus using one cell type only
559 to stimulate an effect or differentiation in the other. Of these qualifying studies, more information on
560 the used cells and culture conditions was extracted and analyzed in Database 2.

561

562 Osteoblasts

563 Osteoblasts are the bone forming cells responsible for depositing mineralized matrix. From all 39
564 studies included in Database 2, the cell types that were present at the start of the co-culture were
565 recorded and are shown in Table 6. More than half used human primary cells, whereas the others
566 used animal primary cells or any type of cell line. Whether OBs or their progenitor cells were applied
567 differed greatly between studies: almost half of the studies started the co-culture with OBs, the others
568 started the co-culture with a type of progenitor cell. It needs to be noted that some of cell descriptions
569 in Table 6 might refer to identical cell populations. This is a result of ambiguous isolation methods and
570 nomenclature which is subjective and can evolve over time (35). This systematic map reflects the
571 nomenclature used by the authors or an unambiguous translation of the provided nomenclature to
572 nomenclature used in this map and does not interpret the provided information if it was ambiguous.

573 One interesting observation regarding the cells used as OBs is that there is little variation in the
574 different types of cells introduced into the co-cultures. Except for the oldest 6 studies that used

575 chicken and rat cells, all studies used human or mouse cells, most of which were primary cells. While
576 the studies using rat and mouse cells mostly directly introduced OBs (either isolated as such or
577 differentiated before seeding), those that used human cells predominantly resorted to using
578 progenitor cells (35). Such OB precursors can be obtained from blood and bone marrow donations
579 and can be expanded to the required number of cells *in vitro*. The main difference between OB versus
580 progenitors is the presence or absence of the osteogenic differentiation phase. Differentiation within
581 the experiment could be desired for the research question or must be considered in case it is not.
582 Those that used primary OBs purchased expandable human OBs (36) or used OBs (37), undefined
583 expanded bone cells (38), or differentiated MSCs (39) from bone material obtained during a surgical
584 procedure.

585 **Table 6: Osteoblast origins and occurrences.**

Cell Origin	Osteoblasts	Mesenchymal stem cells	Mesenchymal stromal cells	Stromal cells	Stromal vascular Fraction	Osteoprogenitor cells	Per-row Total
Human primary	4	9	2	6	1		22
Human cell line	1						1
Mouse primary	3	2					5
Mouse cell line	4						4
Rat primary	3					1	4
Chicken primary				2			2
Reference only	1						1
Total	16	11	2	8	1	1	39

586

587 ***Table 6:*** *From Database 2, the origin of the cells that were used as OB was extracted. Each column*
588 *represents a different cell type of OB-like cells or their precursors. Each row represents a different*

source of cells, differentiating between both the origin species and whether the cells are primary cells or cell lines. Incremental totals are presented in the last row and column.

Seeding densities plays a major role in proliferation and cell function of OBs (18,83). Seeding density of OBs in 2D could be extracted or calculated for 26 studies and ranged from approximately 900 cells/cm^2 to approximately 60.000 cells/cm^2 with a median of approximately 6500 cells/cm^2 and a mean of approximately 11000 cells/cm^2 (Fig 4a). Seeding density of OBs in 3D could be extracted or calculated for 6 studies and ranged from approximately 300 cells/cm^3 to approximately 7*10^7 cells/cm^3 with a median of approximately 4*10^6 cells/cm^3 and a mean of 15*10^6 cells/cm^3 (Fig 4d). It is important to note that these numbers are taken from the entire base of studies in Database 2, and as such are not representative for any type of OB or precursor used. These numbers can be further sorted and selected based on the individual researchers' needs.

Fig 4. Seeding densities and seeding ratios. *Violin plots of 2D and 3D seeding ratios of OB (A+D), OC (B+E) and respective seeding ratios (C+F). Values are calculated based on reported seeding numbers of the cells or precursors thereof by authors per surface are or volume. No distinction was made between different types of cells or precursors in these figures and this introduces a considerable spread in data due to possible cell proliferation (OB) and cell fusion (OC) that might have occurred after seeding. This distinction can be made in the database itself. Please take note that the ranges along the Y-axis are not the same for each figure. Each seeding density of each study is represented by a blue dot.*

Osteoclasts

611 Osteoclasts are the bone resorbing cells that remove old and damaged bone tissue to make place for
612 the deposition of new mineralized matrix. Out of all 39 studies included in Database 2, 20 used human
613 primary cells, the others used animal primary cells or any type of cell line (Table 7). In most cases
614 cultures were initiated with OC progenitors: 16 studies introduced monocytes, 11 introduced
615 mononuclear cells, the rest used other precursors. Again, it needs to be noted that some of these
616 descriptions are ambiguous. What is reported here is the definition used by the authors of the
617 respective studies.

618 The origin of the cells used as OCs is remarkably like those of the OBs. The 6 oldest included studies
619 used chicken and rat cells, and all others used mouse or human cells. With only one exception
620 combining a mouse ST-2 cell line with human monocytes (40), all studies used cells of exclusively a
621 single species for the OB and OC source. Such a similarity was not found regarding the use of cell lines
622 versus primary cells. While many studies introduced OBs directly into the co-culture, only a single
623 study claimed to introduce OCs directly into co-culture but failed to provide any information regarding
624 either cell source and was therefore ignored from further use.

625 Seeding density of OC in 2D could be extracted or calculated for 25 studies and ranged from $5*10^3$
626 cells/cm^2 to $15*10^6$ cells/cm^2 with a median of $42*10^3$ cells/cm^2 and a mean of $190*10^3$ cells/cm^2 (Fig
627 4b). Seeding density of OC in 3D could be extracted or calculated for 6 studies and ranged from $2*10^4$
628 cells/cm^3 to $7*10^7$ cells/cm^3 with a median of $4*10^6$ cells/cm^3 and a mean of $17*10^6$ cells/cm^3 (Fig 4e).
629 Seeding ratios of OB:OC in 2D varied highly and ranged from 1:1500 to 1:1 (Fig 4c) and seeding ratios
630 of OB:OC in 3D ranged from 100:1 to 1:25 (Fig 4f). In human bone tissue, the ratio of OB:OC is
631 estimated to be approximately 7:1 (41). It must be noted that in these numbers, no distinction has
632 been made between the use of precursors versus OB or OC or any type of expansion phase within
633 experiments. These distinctions can be made within Database 2 for each individual need.

634

635 **Table 7: Osteoclast origins and occurrences.**

Cell Origin	Monocytes	Mononuclear cells	Macrophages	Osteoclast precursors	Osteoclasts	Spleen cells	Total
Human primary	10	6	1	3			20
Human cell line	4						4
Mouse primary	2		2	2			6
Mouse cell line			2				2
Rat primary		3				1	4
Chicken primary		2					2
Reference only					1		1
Total	16	11	5	5	1	1	39

636

637 ***Table 7:*** *From Database 2, the origin of the cells that were used as OC was extracted. Each column*
638 *represents a different cell type of OC-like cells or their precursors. Each row represents a different*
639 *source of cells, differentiating between both the origin species and whether the cells are primary cells*
640 *or cell lines. If the cell source was indicated using only a reference, that instance was listed in the row*
641 *'reference only'. Incremental totals are presented in the last row and column.*

642

643

644 ## Co-culture medium composition and culture conditions

645 The behavior of cells is highly dependent on their environment, of which the biochemical part is
646 predominantly determined by the culture medium composition. The main components of typical
647 culture media are a base medium, fetal bovine serum (FBS) and specific supplements such as growth
648 factors, especially when progenitor cells need to be differentiated first. Within the scope of this study,

649 the base medium, FBS content and concentration of typical OB and OC supplements were analyzed. It
650 became obvious that culture conditions are manifold and differ much between studies: A total of 8
651 different base (or complete) media were reported (Fig 5a), with αMEM and DMEM accounting for
652 approximately 80% of all studies. FBS content ranged from 0% to 20%, with most studies using 10%
653 (Fig 5b). Those without supplemented FBS used forms of complete media of which the composition
654 was not described, but possibly including a type of serum or equivalent serum-free supplements.

655 ***Fig 5. Medium components used by studies in Database 2.*** *A) The occurrence of all identified base and*
656 *complete media used during the co-culture phase of each study. B) Serum concentrations during the*
657 *co-culture phase of each study. Numbers report exclusively the use of separately introduced FBS or FCS.*
658 *Serum as part of a complete medium kit that was not described in the methods section is not reported*
659 *here. C) OC supplements administered during the co-culture phase of each study. OC supplements were*
660 *exclusively reported in ng/ml and are reported as such in the violin plot with all individual*
661 *concentrations as blue dots. Please note that the x-axis has a linear distribution. D) Osteogenic*
662 *supplements during the co-culture phase of each study. Osteogenic supplements were recalculated to*
663 *molarity where necessary for comparability. Individual molarities are shown as blue dots. Please note*
664 *that the x-axis has a logarithmic scale.*

665

666 OC supplements were reported exclusively in ng/ml (Fig 5c). M-CSF concentration was reported in 11
667 studies and ranged from 10 ng/ml to 100 ng/ml with a mean of 39,82 ng/ml. RANKL concentration
668 was reported in 14 studies and ranged from 10 ng/ml to 100 ng/ml with a mean of 49 ng/ml. All OB
669 supplements were recalculated to molarity if they were reported in concentrations (Fig 5d). Ascorbic
670 Acid (AA), which was also referred to as ascorbic acid-2-phosphate, L-ascorbic acid or L-ascorbate-2-
671 phosphate, was used in 19 studies. AA concentration ranged from 0.05 mM to 0.57 mM, with mean
672 of 0.18 mM and one outlier at 200 mM that was disregarded for this calculation. Dexamethasone was
673 used and reported in 13 studies and was used in 2 different molarities: 6 times at 10^{-7} M and 7 times

674 at 10^{-8} M. β-Glycerophosphate (βGP) use was reported in 17 studies, and ranged from 1 mM to 46
675 mM, with a mean of 13 mM.

676

677

678 ## Other culture conditions and techniques

679 In addition to the cell and medium characteristics, there are other factors that define an experiment.
680 Out of the 39 studies of Database 2, 9 studies used a type of transwell or well insert culture (where
681 cell populations are separated, but factor exchange is possible), 16 studies used a form of 3D culture,
682 3 studies reported the use of bioreactors, 2 studies used more than the required 2 cell types to form
683 a tri- or tetra-culture (39,42), 7 studies reported using non-standard environmental conditions such
684 as gas concentrations or mechanical loading. Polymerase chain reaction (PCR) was used in 13 studies,
685 and supernatant analyses such as ELISA were used in 14 studies. The target genes, proteins or
686 compounds were extracted from the publications and the occurrence of each target was recorded in
687 the analysis of Database 2.

688

689 Discussion

690 In recent years, many research groups have ventured into the realm of OB-OC co-cultures with the
691 intent of studying both formation and resorption. Due to a lack of standardization within the field and
692 the difficulty of finding publications based on methods instead of results, each group seems to be
693 individually developing the tools to suit their needs resulting in many functionally related experiments
694 that are methodologically completely different. The use of OB-OC co-cultures is usually not clearly
695 mentioned in the title and abstract, making it difficult to find these studies without a systematic search

and thorough review. The aim of this study was to generate a systematic map to give an overview of existing osteoblast-osteoclast co-culture studies published up to 6 January 2020, and present their methods, predetermined outcome measures and other useful parameters for analysis in 2 databases which can be filtered, sorted, searched and expanded.

The Database 1 contains all OB-OC co-culture studies in which at least one relevant primary outcome measure (formation and/or resorption) or secondary outcome measure (ALP and/or TRAP quantification as surrogate markers for formation and resorption, respectively) was investigated (S1_File_Database_1). A sub-selection of studies that have relevant outcome measures investigated on both OBs and OCs in the co-culture are shown in Database 2, accompanied by additional details on methods, culture conditions and cells (S2_File_Database_2).

Resorption

Most studies in Database 1 investigating resorption did so in 2D cultures using a resorbable substrate such as bone, dentine, or synthetic osteological discs. This is not unexpected, as these three options are either the actual *in vivo* material (bone), a similar material with excellent properties for studying resorption (dentine) (43), or a material designed specifically for the purpose of studying resorption (osteologic discs or coated wells). One crucial advantage of using dentine discs over bone is related to the native structure of dentine itself: it does not contain canaliculi and has fewer other irregularities because it is not actively remodeled, providing more contrast between the native structure and resorption pits to accurately visualize them (43,44). Because of that reason, dentine is often favored over bone. The advantages of bone over dentine are that bone is the actual tissue of interest as opposed to a bone-like material, it can be obtained from many different species in relevant quantities and sizes, can be more easily be prelabeled *in vivo* with for example radioactive markers such as 3H-proline (45), it is cheaper and more readily available, and could be used in conjunction with cells from

720 the same species or even same animal, although the latter was not observed in this map. Dentine is a
721 component of ivory, usually obtained from elephants (46), hippo's (47) or sperm whales (48).
722 Regulations regarding ivory are strict and the material is rare, making it difficult and expensive to
723 import and obtain. Synthetic osteologic discs have the advantage of being produced in a uniform
724 manner and should show little sample-to-sample variation compared to discs made from animal
725 tissue, or hand-made discs. Using well plates with thin osteologic coatings has the advantage that once
726 the coating is resorbed, the translucent well below is revealed, which facilitates imaging with light
727 microscopes. Combined with certain stainings, it makes quantifying resorbed area using conventional
728 light microscopy easier.

729 Choosing the surface that will be resorbed by the osteoclasts will result in a compromise. For example,
730 HA and other calcium phosphates are a likely choice for studying resorption since they are a major
731 constituent of bone. While not optimized to facilitate resorption per se, they are simple to create,
732 have a known composition and should offer good between-lab reproducibility. This contrasts with
733 resorbable discs and plates with undisclosed ingredients and likely between-manufacturer variation.
734 They are however synthetic, and do not contain any organic ECM components, which means that
735 techniques such as measuring bone turnover markers NTx (49) and CTx (50) do not work.

736 It is believed that the deposition of collagen type I by osteoblasts is a vital step in the formation of
737 mineralized tissue (51), and similarly could play a role in the resorption thereof. It is also possible to
738 generate the to-be-resorbed material *in vitro* by the OBs (50), even within the same experiment. This
739 essentially simulates a bone remodeling environment that is a step closer to the physiological process
740 of bone remodeling versus only resorption, although *in vivo* the order in which this typically happens
741 is reversed: first, damaged ECM is resorbed by OC, then new ECM is deposited by OB (52). However,
742 the process of creating a mineralized matrix may introduce a variation in substrate size even prior to
743 initiating the co-culture (53). Also, many *in vitro* formation experiments, while being able to produce
744 the ECM constituents collagen and mineral, are not (yet) producing real bone ECM (51). An advantage

745 specific to using a collagen-based material in favor of a pure ceramic material is that techniques such
746 as NTx (49) and CTx (50) can be used. These bone turnover markers are used in the clinic and can
747 quantify resorption by directly analyzing the liberated collagen fragments that were present in the
748 resorbed mineralized matrix (54).

749 Because most studies were conducted in 2D, most resorted to using various types of 2D microscopy
750 to analyze resorption, usually after staining to increase contrast. This can facilitate the quantification
751 of resorbed area using image analysis software but is usually limited to a quantification of surface
752 area, whereas resorption is a three-dimensional process. While methods exist to reconstruct a set of
753 stereoscopic 2D images into 3D height maps (55), these were not identified within the studies in either
754 database of this systematic map. It would be better to consider imaging techniques that can directly
755 quantify the resorbed volume. Examples are 2-photon microscopy for thin samples and micro
756 computed tomography (µCT) (56). Due to the non-destructive nature of µCT, it is well suited to
757 monitor mineralized volume over time within the same samples (57) and images can be compared for
758 changes over time (53,56). The usefulness of such a monitoring tool is however dependent on the
759 envisaged resolution versus the corresponding potential cell-damage caused by radiation exposure
760 (58,59). Registering consecutive images can even show both formation and resorption of mineralized
761 tissue within the same set of images of the same sample if both mineralizing OBs and resorbing OCs
762 are present (53). While µCT in this map is predominantly used on 3D samples, one study used it to
763 quantify the thickness of mineralized films and combined that data with surface metrological data
764 (60).

765 Overall, the golden standard (bone and dentine discs) remains the most-used method to study 2D
766 resorption, although alternatives such as osteological coatings offer new and easy ways of
767 quantification. Compared to 2D cultures however, 3D cultures are under-represented in this
768 systematic map. While the systematic search covers all publications until January 6 2020 available,
769 only 24 studies were labeled as 3D co-cultures in Database 1, the first being published only in 2006

(61). From these we learn that studying 3D resorption remains a challenge, with the only identified viable options for quantification being µCT imaging and supernatant analysis techniques such as NTx and CTx.

Formation

The result of bone formation is the deposition of mineralized matrix. This is however a multi-step process of the presence of properly stimulated OBs that lay down a framework of type I collagen, which in turn is mineralized by the addition of calcium phosphates (51). No single method of measuring formation confirms the occurrence of each step in this process, instead relying on the assumption that the confirmed presence of one step indicates the presence of the entire process.

With most studies being two-dimensional co-cultures, it is no surprise that most formation analyses extracted from Database 1 were stainings. Of these, Alizarin Red is particularly interesting due to the option of quantifying the amount of bound dye, which correlates to the amount of calcium (27). A risk when using this method on larger samples is that it is not certain how far both dye application and dye extraction penetrate the material. This should not affect relative comparisons between different sample groups but could lead to underestimations of calcium deposition. By completely lysing the samples and directly measuring the exact amount of calcium or phosphate (62,63) this risk could be avoided, at the cost of not gaining information on the location and distribution of calcium or phosphate through the sample.

The two types of non-destructive formation measurements, CICP and µCT, are coincidently well-suited for the analysis of three-dimensional co-cultures as well. A major advantage of these is that because of their non-destructive nature, they can be used to measure the same samples over time, and they can be used prior to other destructive techniques. CICP measurements (64) have no negative effects on the co-culture, requiring only that culture supernatant samples can be taken at the desired

timepoints, usually at medium exchange. The use of µCT leads to both quantification and visualization of mineralization within the same sample over time, but it has some aspects to consider. Most importantly, to use it as a non-destructive technique the samples must be cultured in sterile vessels capable of being scanned. This means that experiments are limited by severe practical constraints. Additionally, there is a direct correlation between the resolution of the images (and thus the minimal detectable size of mineral deposits) and exposure to radiation and subsequent cell damage (58,59). Radiation damage directly affects the usefulness as a monitoring tool, and a careful balance between minimal acceptable resolution and maximal radiation exposure must be found.

Overall, 2D nodule stainings were the most frequently used method to measure formation. Combined with Alizarin Red dye release these provide an easy way to quantify mineralization, though CICP supernatant analysis and µCT techniques provide a non-destructive alternative that can also be used for 3D co-cultures.

ALP and TRAP

ALP and TRAP are the two major markers used for indirectly quantifying respectively OB and OC activity that were included into Database 1. Their presence is no conclusive proof that formation and resorption are occurring because ALP is expressed in differentiating MSCs already (65) and TRAP is expressed on monocytes as well (53), but there is a correlation between their presence and that of OB and OC, respectively. ALP is an enzyme that makes phosphates available to be incorporated into the matrix (66), while TRAP has been associated with migration and activation of OC (67). These enzymes can be measured both after lysis of the cells or within the culture supernatant. The former allows the quantification of enzyme per DNA content when combined with a DNA assay, whereas the latter allows the monitoring of relative enzyme release over time. The precise methodological details and experimental setup are of lesser importance for measuring ALP and TRAP than they are for

818 measuring formation and resorption. All that is required is the possibility to use the supernatant or
819 cell lysate, which is possible in most common experimental setups. The most frequently used methods
820 are the pNPP-based methods where ALP and TRAP directly convert a substrate into a measurable
821 compound. Napthtol-based methods (29) rely on a similar principle, and show an increased specificity
822 for TRAP isoform 5B in particular (30). The main advantage of these methods is that they use the
823 inherent enzymatic activity of ALP and TRAP, reducing the complexity and cost of the assay. However,
824 the reliance on the inherent enzymatic activity of the enzymes is also a practical limitation as inherent
825 activity can be affected by freezing and long-term storage. Especially when monitoring ALP or TRAP
826 release over time, samples are commonly frozen and stored for different periods of time, and enzyme
827 activity could be affected by this. A workaround would be to directly analyze the samples after taking
828 them, or to use methods that rely on the presence and not the activity of these enzymes.

829 One of those methods is the immunoenzymatic assay, of which ELISA is the most well-known. With a
830 traditional ELISA the antigen is first bound to the assay plate, and then labeled with one or a series of
831 antibodies that are conjugated with an enzyme to convert a substrate to a chromogenic product (68).
832 These methods have the capacity to detect lower concentrations of protein because it is possible to
833 label each individual protein with an excess of new enzymes each capable of converting substrate. In
834 the case of TRAP, ELISA kits exist that are specific for TRAP isoform 5b which is expressed almost
835 exclusively in OCs (69), whereas isoform 5a is also expressed by macrophages and dendritic cells (70).
836 While in a co-culture with pure populations of OB and OC this distinction would not be relevant,
837 macrophages or macrophage-like cells can be used as a precursor for OCs (24), and thus express
838 isoform 5a which could be detected in a pNPP based assay. Similarly, most co-cultures use a precursor
839 or heterogeneous population that either contains macrophages or contains cells capable of
840 differentiating into macrophages such as mononuclear cells (71), which means that the presence of
841 other isoforms or even other phosphatases is likely. Whether this negatively affects the results is
842 another matter that can only be determined by comparison between the two types of assay. Another
843 factor to consider in co-cultures is the fact that both ALP and TRAP are phosphatases. Assays that rely

844 on their inherent phosphatase activity may show cross-reactivity of other phosphatases, although this
845 should be mitigated by controlling the pH during the test.

846 To conclude, pNPP based methods are the most frequently used methods for detecting ALP and TRAP
847 due to their affordability and simplicity. However, immunoenzymatic detection methods are more
848 sensitive and specific, and do not rely on the intrinsic enzymatic activity of ALP and TRAP which can
849 be affected by freeze-thaw cycles, long-term storage, and could show cross-reactivity with other
850 phosphatases.

851

852 Osteoclasts

853 OCs are the bone resorbing cells, and together with bone forming OBs they keep the bone mass and
854 bone strength in equilibrium with the required loads placed upon it. OCs are created when OC
855 precursors such as monocytes exit the bloodstream because of chemotactic cues followed by the
856 correct biochemical signals that result in cell-fusion into OCs. Cells are currently considered to be OCs
857 when expressing TRAP, having an actin ring, and having at least 3 nuclei (6). Osteoclastic resorption *in*
858 *vivo* is an integral part of bone maintenance. Old and damaged bone tissue is resorbed and quickly
859 replaced by OBs with new bone tissue.

860 There is a clear preference in the studies identified for Database 2 for using human cells to generate
861 OCs, most notably monocytes and mononuclear cells. These have in the past two decades proven to
862 be a reliable and relatively straight-forward precursor population for OCs (6), they can be obtained
863 from human blood donations, and are thought to be better representatives for studying human
864 physiology than cells of animal origin (2,3).

865 The choice of using precursors versus differentiated OCs is forced sharply into one direction because
866 of both biological and experimental limitations. The extraction of OCs from bone is possible but

867 cumbersome, requires access to fresh bone material and generally does not yield relevant numbers
868 of OCs. Generating OCs from circulating precursors has proven to be an easier way of obtaining OCs.
869 However, OCs have an average life span of approximately 2 weeks (72,73), some of which would
870 already be lost if OCs would be created prior to the actual experiments. In contrast to most cells,
871 differentiation happens by fusion of several precursors into a single OC. Fused multi-nucleated OCs
872 can become large and hard to handle without damaging them. For those reasons they are usually
873 generated within the experiment itself instead of in a prior culture. In fact, the first OB-OC co-cultures
874 were designed specifically to generate OCs by using osteoblastic cell signals (9), as opposed to
875 generating a model to study both OBs and OCs simultaneously as this systematic map has indexed
876 (74).

877 OCs can currently be obtained *in vitro* without the need for OBs thanks to the discovery in 1999 that
878 M-CSF and RANKL are the necessary and sufficient proteins to induce osteoclastic differentiation from
879 precursors (10). The cells are predominantly introduced into the co-culture as precursors to
880 differentiate within the co-culture, regardless of whether these two proteins are used or not. Where
881 in the past researchers used spleen cells for this, the studies included in this systematic map
882 predominantly use (blood-derived) mononuclear cells, monocytes, or macrophages as precursor cells.
883 These four sources are closely related, and the main differences between them are the purity of the
884 population and how far along the path to differentiated and active OCs they are. In short: Spleen cells
885 contain many cells, among others mononuclear cells. A part of the mononuclear cell population
886 consists of monocytes which are currently regarded as 'the' OC precursors (75,76). Monocytes can
887 differentiate into macrophages or fuse together into OCs, depending on the biochemical cues
888 received. Macrophage-like cell-lines are being used to generate OCs as well.

889 There are risks associated with each method of generating OCs. Animal cells introduce a between-
890 species variation and can respond differently than human cells (17), human donor cells tend to exhibit
891 large between-donor variation compared to cell lines (77) and the number of cells acquired is limited

and variable (78). The large variation between donors again highlights the need for patient-specific disease models instead of generic bone models. By using cells of a single diseased donor, the reaction of that patient's cells on potential treatment options can be studied. Immortalized cell-lines result in immortal subsequently generated OC-like cells. This is however not the case *in vivo* and while it can greatly reduce between-experiment and between-lab variation, it is also physiologically less relevant. While these risks and characteristics do not discredit any source as a viable source of OCs for any experiment, the results of the corresponding studies should be interpreted with these characteristics in mind.

Osteoblasts

OBs are the bone forming cells, and together with bone resorbing OCs they keep the bone mass and bone strength in equilibrium with the required loads placed upon it. In addition to their role in bone formation, they excrete the exact biochemical cues necessary to generate OCs out of their circulating precursors. Before the identification and commercial synthesis of these factors, a co-culture with OB was the only way to generate OCs *in vitro*.

The preference for the use of human primary cells identified in the studies included in Database 2 can be explained by the good availability of donor material, expandability of OB precursors, and because human cells have the potential to better reflect human physiology than cells from other species (2,3). The choice of OB progenitors versus OBs is not as crucial here as it is with OCs. MSCs, the most commonly used precursors, have a tri-lineage potential (79) and should be able to differentiate into OBs on a 1-1 ratio. The advantage of osteoprogenitors such as MSCs is that these are capable of extensive proliferation before differentiation and could be used to migrate into and populate hard-to-reach areas within 3D scaffolds. Additionally, using progenitors opens possibilities to study osteoblastogenesis in addition to bone formation. When the effect of an intervention on

916 mineralization but not osteogenesis is under investigation, care must be taken that the intervention
917 is not applied before differentiation is has been achieved.

918 The advantage of directly introducing OBs instead of precursors, whether obtained directly from
919 primary material or pre-differentiated *in vitro*, is that these do not need to be differentiated within
920 the experiment anymore, and all seeded cells are already OBs, and by extension, any experimental
921 conditions affect only mature OBs and not osteoblastogenesis in parallel. Actual OBs or to-be-
922 differentiated MSCs isolated from orthopedic surgery are the most common source of primary human
923 OBs. However, healthy human donor OBs are scarce because they are mostly isolated after surgery of
924 mainly diseased patients. Whether the use of OBs from unhealthy donors affects experimental results
925 needs to be elucidated. On the other hand, using patient cells to create a personalized *in vitro* disease
926 model is the first step towards personalized medicine, especially if all cells are of that same patient.
927 Finally, the use of any type of animal cell instead of human cells carries the risk of finding inter-species
928 differences that can affect the results and conclusions, and everything based on that, because animal
929 cells can behave differently than human cells (17). While none of these risks directly discredit any of
930 the methods obtaining OBs, the results must be interpreted with these risks and characteristics in
931 mind.

932

933 Culture conditions

934 The success of a cell-culture experiment is dependent on many factors related to culture conditions.
935 For most cell-types, standard culture conditions have been established. During co-culture experiments
936 however, the needs of two or more cell types need to be met. Medium components and factors may
937 be needed in different concentrations, as they can be beneficial to one cell type but inhibitory to the
938 other (80).

There is a clear preference for medium based on DMEM and αMEM, but the choice of base medium for a culture is not an easy one. Base media are generally chosen based on the intended cell type, recommendations by a manufacturer or supplier of either cells or medium, preferred effect on cells, interaction with other supplements, and earlier experience. These factors make direct comparison of experimental results by literature virtually impossible. Additionally, none of the studies mentioned why they specifically chose the base media they used.

Another variable in medium composition is the use of FBS (or FCS). It is commonly known that there can be batch-to-batch and between-brand differences in FBS (81) which can impact the results of an experiment tremendously. While different concentrations are being used, the most common FBS concentration is 10%. However, no study explains why each type and concentration of FBS was used.

Although there was no clear predictor for using or not using any of the osteoblastic or osteoclastic supplements, when they were used, the concentrations were usually within the same order of magnitude in all studies, except for ascorbic acid. However, only 2 studies used all 5 of the supplements indexed in this study (AA, βGP, Dexamethasone, M-CSF and RANKL) and many combinations of supplements have been registered in this map. Looking at OC supplements, it is generally accepted that RANKL and M-CSF are both necessary and sufficient for osteoclastogenesis (10). However, OBs can produce RANKL and M-CSF themselves to trigger differentiation (9) and therefore the supplements are not necessarily required in co-culture. The need for all osteoblastic supplements is not as great considering osteoblasts can be introduced in various stages of development. Still, each supplement contributes to a specific function. Dexamethasone upregulates osteogenic differentiation, βGP acts as a phosphate source, and AA is a co-factor involved in collagen synthesis (82). Depending on the type of cells introduced, the aim of the experiment and other methodological details, their inclusion could be beneficial.

Finally, many studies used or omitted specific supplements related to their research question regarding the activity of OBs or OCs or used less common supplements for differentiation such as

964 vitamin D3, human serum or Phorbol 12-myristate 13-acetate. What is seldom addressed however, is
965 the compromise that must be made in choosing the right supplements and concentrations. Adding
966 too high doses of supplements could cause an excess of these signals in the culture medium,
967 effectively overshadowing any other ongoing cell-signaling over the same pathway by other cells. This
968 is of critical importance when the goal is not to achieve only OBs and/or OCs activity, but to obtain a
969 homeostasis in which the two cell types regulate each other, with experimental conditions or
970 interventions that are expected to affect this balance. Here, it may be beneficial to experiment with
971 lower concentrations of factors, supplemented only during critical phases of the cells' development
972 or differentiation.

973 The choice of medium in a co-culture is most likely going to be a compromise and must be based on
974 the exact research question to be addressed, where the advantages and disadvantages of base media
975 and supplements for both cell types are carefully weighed. Most likely, the ultimate goal for the
976 envisaged co-culture would be to reach tissue homeostasis, in which the environment is as similar to
977 the *in vivo* environment in tissue homeostasis where cell interactions with each other can be
978 monitored.

979

980 Seeding densities and seeding ratios

981 Using the correct seeding densities plays a major role in proliferation and cell function of OBs (18,83)
982 and osteoclastic differentiation (84). The seeding densities reported in this map show an enormous
983 spread. Many factors could have influenced these numbers. For example, some studies report the
984 numbers prior to expansion, others expand the cells in (co-)culture. Similarly, the percentages of
985 relevant precursor cells in heterogenous cell populations can vary widely. The cell numbers present
986 and OB:OC ratio most likely even change during a co-culture due to ongoing cell-division,
987 differentiation, fusion and different expected life spans and the corresponding cell death. Regrettably,

the available documentation of exact cell numbers introduced is often lacking, and open to some interpretation. While the figures show this large spread in data points, the included databases can be manipulated to filter and select studies that match criteria according to the readers' specific needs.

Animal type, cell type, cell line versus primary cells and even passage number may also directly influence the choice of seeding densities in addition to various experimental choices. At the same time, the purpose of the experiment and more specifically the purpose of the cells and type of interaction required should determine the necessary seeding density. Are the cells required to actively deposit or resorb measurable amounts of minerals, or are they just supposed to be there to facilitate OB-OC communication? The combination of all these factors suggests that there in fact is no one ideal seeding density, that the best density for a certain experiment can only be determined by taking all the above factors into account, learning from others that did a similar experiment, and most importantly verifying assumptions and predictions in the lab.

Looking at the cell seeding ratio, here reported as number of seeded OB/OB-precursors per seeded OC/OC-precursor, outliers can be normalized against their seeded counterparts. In 2D studies, there are never more OBs/OB-precursors than OCs/OC-precursors. At most, they are seeded at a 1:1 OB:OC ratio. Even though in human bone tissue the ratio of OB:OC is estimated to be approximately 7:1 (41), higher OC numbers than OB numbers are not unexpected. OB precursors can still proliferate, whereas OC precursors usually still need to fuse together to form mature OC or OC-like cells. In 3D we do not see the same trend, with ratio's ranging from 1:20 to 100:1. These differences are again affected by the same factors that influence individual OB and OC seeding densities, further enhanced by the extra layer of complexity that are inherent to 3D cultures.

Limitations of the systematic search

While the authors took great care to construct a series of search queries fine-tuned for each of the three online bibliographic literature sources, the authors cannot be certain that all relevant OB-OC co-cultures have been included into the two databases. The search was limited by the necessary addition of a 'co-culture' search element. Co-culture studies without any indication thereof in the title or abstract simply cannot be identified through the initial search. To compensate for this, screening step 4, searching through identified reviews and publications included into Database 2, was executed. The publications included into Database 1 or the complete list of identified OB-OC co-cultures could have been screened for references as well, but the authors decided against this. Database 2 was specifically chosen for this because the likelihood of a publication that matches all relevant inclusion criteria citing other such publications was deemed high, whereas less relevant papers (included into Database 1, or not included at all) were considered much less likely to cite publications relevant to this systematic map that had not already been identified by the search itself or the screening of reviews and Database 2. Publications in languages other than English, Dutch or German were excluded because none of the researchers involved in data curation and analysis were fluent in those languages. No budget was available to hire a professional translator for the remaining languages. The consequence of that is that there is a likelihood that relevant publications were missed.

Limitations of the databases

The use of co-culture models is a field that is still developing, and we are now aware that it is not only about adding an additional cell type, but that the complexity of such a culture is more than just doubled. The applied choice of methods, cells, and culture conditions should be tailored to the research question to be investigated, and ideally would be comparable to other studies within the field. This systematic map shows that the currently applied methods are far from standardized and that many research groups have developed their own approach attempting to overcome each

challenge, making comparison between research groups virtually impossible. There is no consensus on cell types, seeding densities, seeding ratios or medium composition, and many of these are predominantly determined by the research question and whatever has been done before in each respective laboratory. For each study, 86 columns worth of data has been extracted including in some cases extrapolation and recalculation of numbers, which are now available for sorting and filtering for individual needs. Still these databases only scratch the surface of each study, and to fully understand the collected information and the context on which it was gathered, one must still read the full publication.

It must be noted that the quality of reporting in many cases is lacking. Both missing information critical and non-critical for reproducing the methods of the studies was identified, and only 13 out of 39 studies included in Database 2 did not miss at least a basic description of all indexed characteristics. However, more relevant details of these characteristics may have been omitted that describe exactly how each method or culture conditions was executed that were not required for this systematic map. Instead, this systematic map focuses on a high-level indexing and evaluation of defining characteristics of methods and culture conditions.

This systematic map is not intended to provide a definitive answer to the question of how to set up the perfect OB-OC co-culture. Instead, it allows searching through all relevant co-culture studies looking for specific matching experimental characteristics or culture details that may be applicable to one's own research. For this, it contains the possibility to search, sort and filter through many relevant characteristics. This allows one to find relevant studies that may have already (partly) studied one's research question, or that can be used as a guide to design comparable experiments.

Conclusion

With this systematic map, we have generated an overview of existing OB-OC co-culture studies published until January 6, 2020, their methods, predetermined outcome measures (formation and resorption, and ALP and TRAP quantification as surrogate markers for formation and resorption, respectively), and other useful parameters for analysis. The two constructed databases are intended to allow researchers to quickly identify publications relevant to their specific needs, which otherwise would have not been easily available or findable. The presented high-level evaluation and discussion of the major extracted methodological details provides important background information and context, suggestions and considerations covering most of the used cell sources, culture conditions and methods of analysis. Finally, this map includes the instructions for others to expand and manipulate the databases to answer their own more specific research questions.

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Supporting information

S1 File. Database 1. This database contains all studies in which at least one relevant outcome measure was investigated. Characteristics of outcome measures and descriptive statistics are listed in this database.

S2 File. Database 2. This database contains all studies in which at least one relevant outcome measure was investigated for both OB and OC. Characteristics of cells, methods and culture conditions, and descriptive statistics are listed in this database.

S3 File. Using the databases. This document provides instructions on how to operate the databases, how to add publications and expand the analyses with more elements.

S4 File. List of all OB-OC co-cultures. This list contains the initial list of 694 OB-OC cocultures obtained after screening, before full-text investigation and exclusion based on outcome measures.

S5 File PRISMA checklist. The PRISMA checklist describing all elements of the systematic review, and on what page or which section of the submitted manuscript to find them.

S6 File Systematic Review Protocol and Search Queries. The protocol and search queries as they were published prior to execution of the fulltext screening phase.

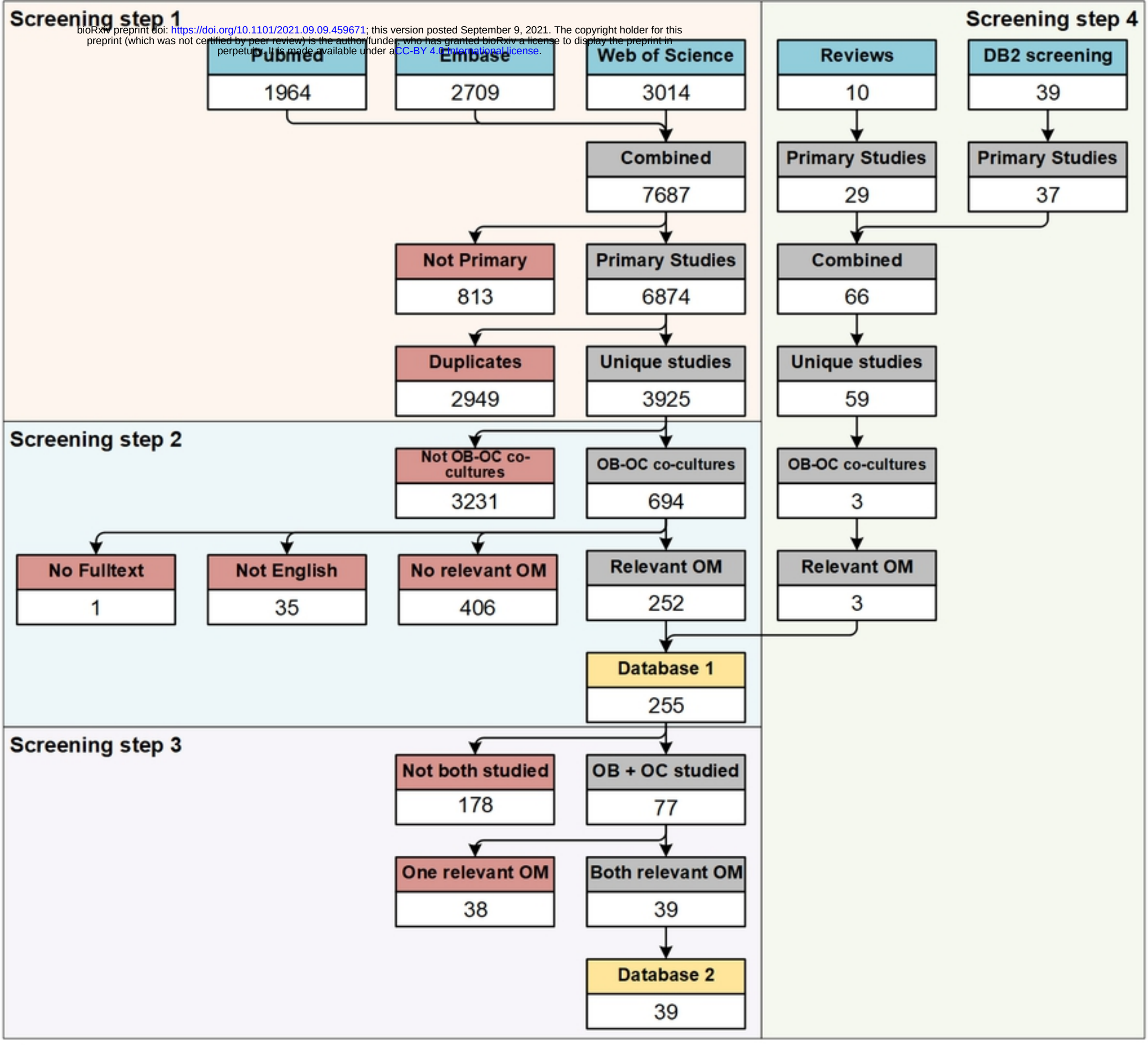
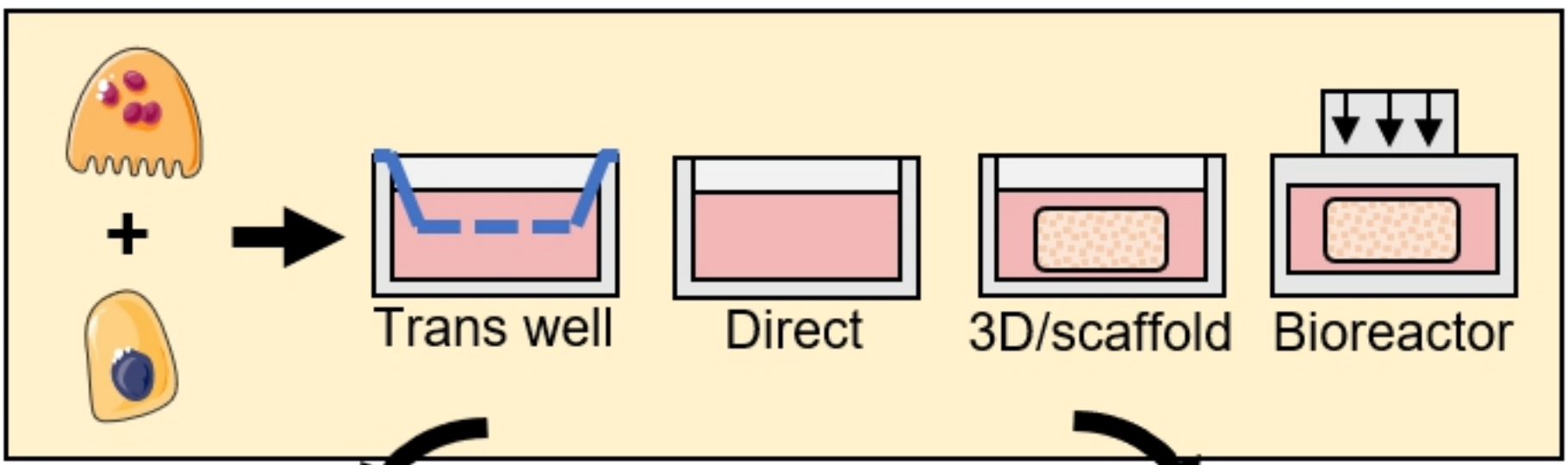


Figure 1

Systematic literature search

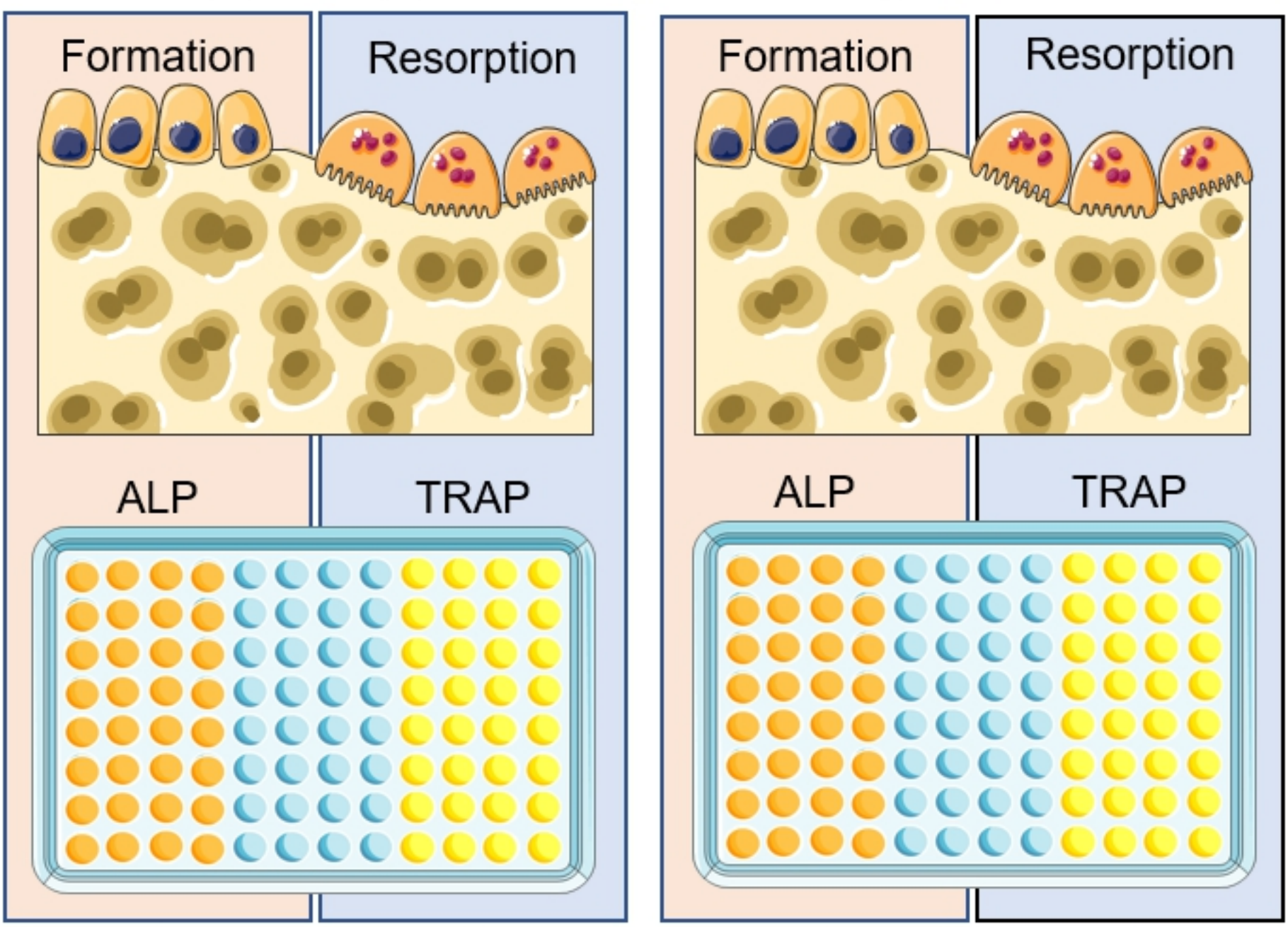
Identification of cocultures



Database 1

Database 2

Primary outcome



Secondary outcome

Or

And

Data extraction

Resorption or formation related outcome measures	Resorption and formation related outcome measures and culture conditions

Figure 2

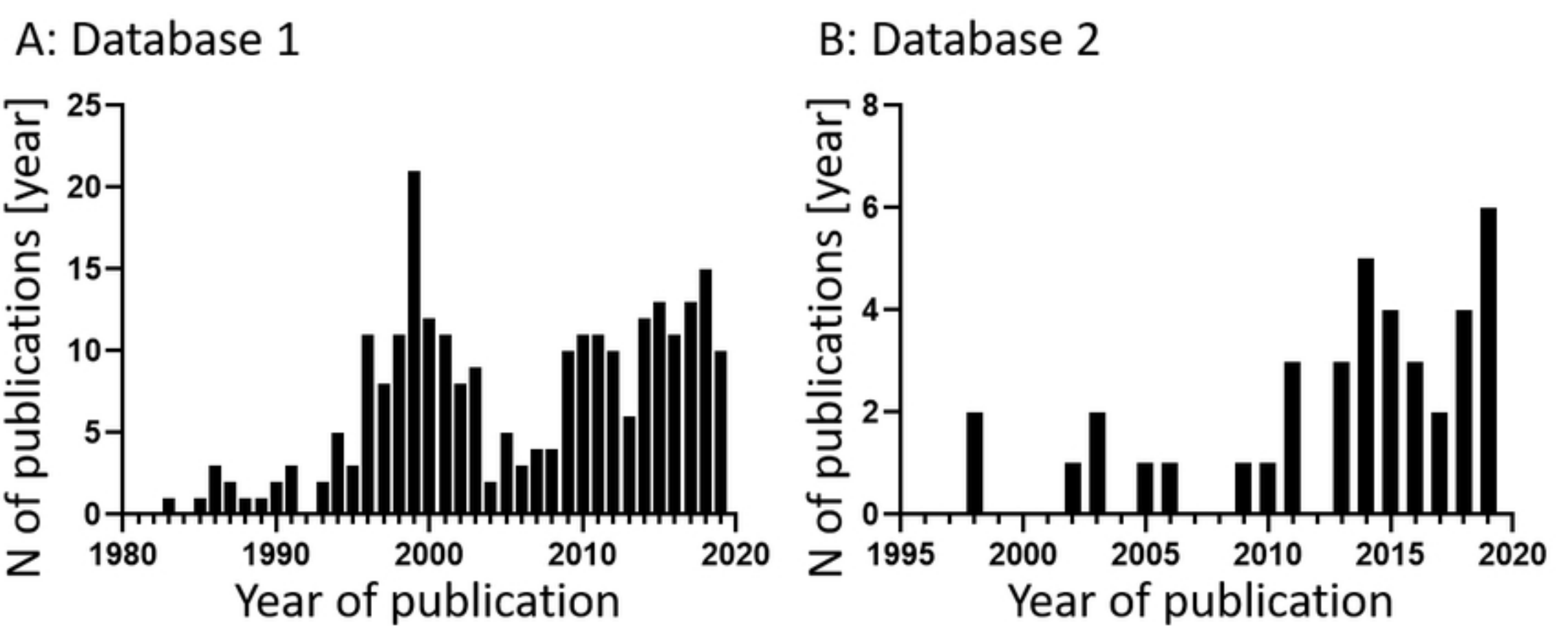

A: Database 1
N of publications [year]
25
20
15
10
5
0
1980
1990
2000
2010
2020
Year of publication
B: Database 2
N of publications [year]
8
6
4
2
0
1995
2000
2005
2010
2015
2020
Year of publication

Figure 3

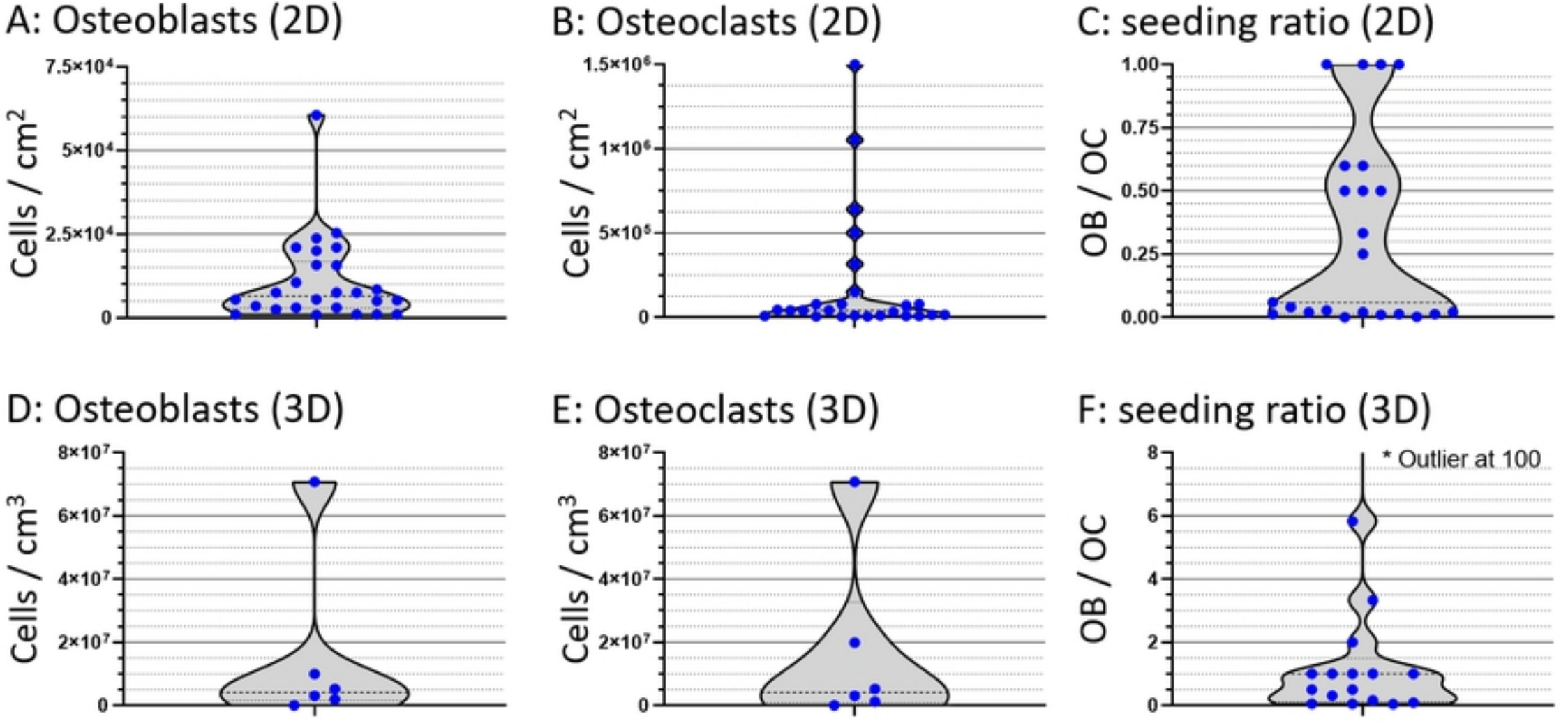

A: Osteoblasts (2D)
Cells / cm2
7.5×10⁴
5×10⁴
2.5×10⁴
0
B: Osteoclasts (2D)
Cells / cm2
1.5×10⁶
1×10⁶
5×10⁵
0
C: seeding ratio (2D)
OB / OC
1.00
0.75
0.50
0.25
0.00
D: Osteoblasts (3D)
Cells / cm3
8×10⁷
6×10⁷
4×10⁷
2×10⁷
0
E: Osteoclasts (3D)
Cells / cm3
8×10⁷
6×10⁷
4×10⁷
2×10⁷
0
F: seeding ratio (3D)
OB / OC
* Outlier at 100
8
6
4
2
0

Figure 4

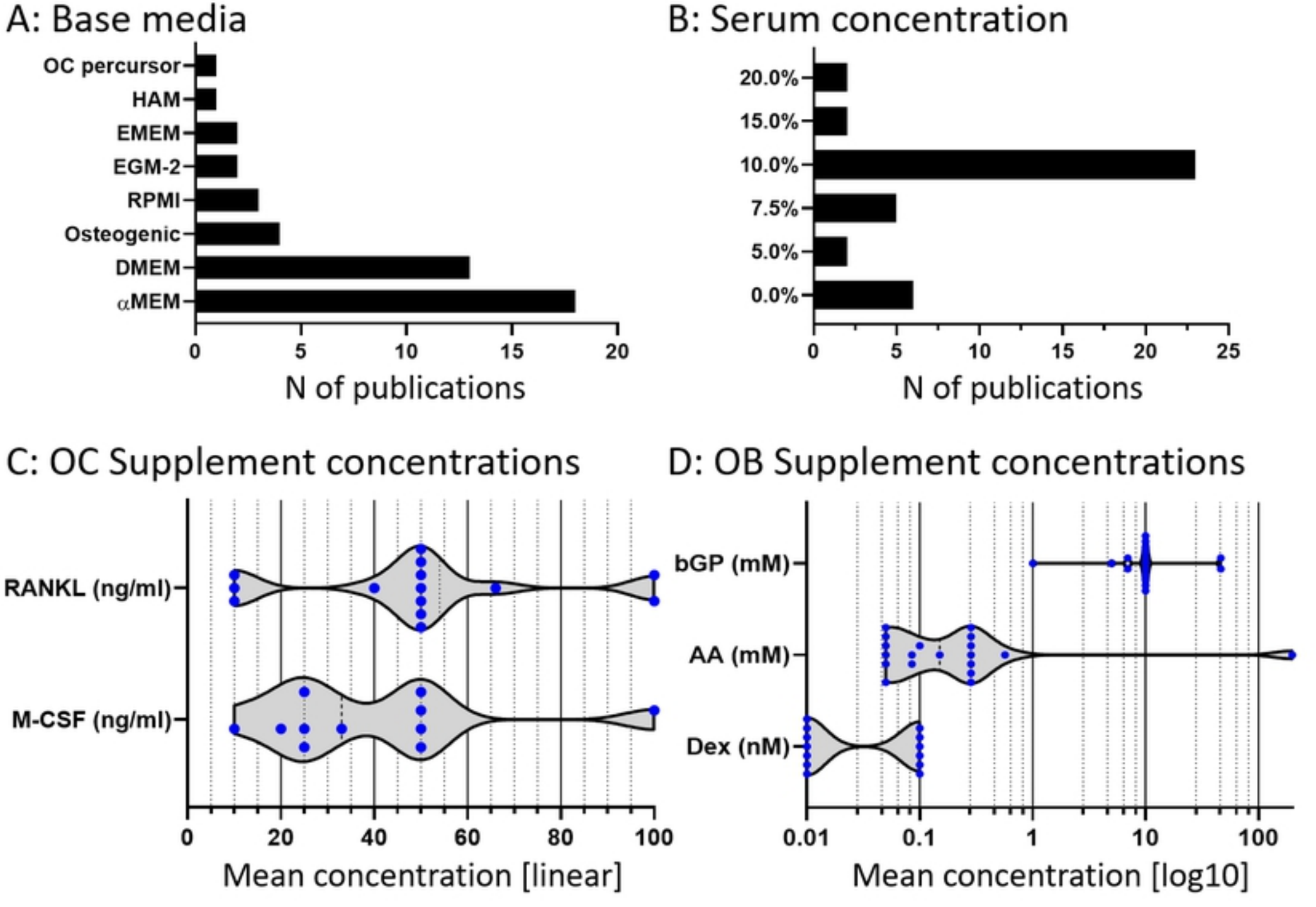

A: Base media
OC percursor
HAM
EMEM
EGM-2
RPMI
Osteogenic
DMEM
αMEM
0
5
10
15
20
N of publications
B: Serum concentration
20.0%
15.0%
10.0%
7.5%
5.0%
0.0%
0
5
10
15
20
25
N of publications
C: OC Supplement concentrations
RANKL (ng/ml)
M-CSF (ng/ml)
0
20
40
60
80
100
Mean concentration [linear]
D: OB Supplement concentrations
bGP (mM)
AA (mM)
Dex (nM)
0.01
0.1
1
10
100
Mean concentration [log10]

Figure 5