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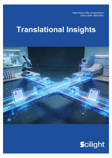

Article

Dissecting the Transcriptional Variability of Breast Cancer-Associated Fibroblasts Using Different Culture Conditions

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Abstract: The tumor microenvironment plays a critical role in breast cancer (BC) progression, with cancer-associated fibroblasts (CAFs) representing a highly plastic stromal population. Although primary CAFs are widely used to study tumor biology, the extent to which experimental conditions impact on their molecular identity and phenotype remains to be elucidated. Here, we investigated how culture conditions may influence the properties of CAFs isolated from female and male BC tissues. CAFs maintained under diverse culture conditions displayed marked differences in morphology and proliferation, indicating that *in vitro* environments may influence the cellular behavior. Accordingly, transcriptomic analysis revealed that culture conditions may represent a major determinant of CAFs identity, shaping distinct gene expression programs. Specifically, depending on medium composition, CAFs activated pathways associated with lipid metabolism or programs related to extracellular matrix organization. Collectively, our findings suggest that culture conditions may shape CAFs functional states, underscoring the need for careful standardization to ensure robust interpretation and enhance the translational relevance of studies using tumor tissue-derived cells.

Keywords: CAFs; primary cells; breast cancer; RNA-sequencing

1. Introduction

Breast cancer (BC) is a highly heterogeneous disease and remains one of the leading causes of cancer-related mortality worldwide [1]. Beyond the intrinsic diversity of malignant cells, increasing evidence indicates that cancer progression is influenced by the tumor microenvironment (TME), which plays a central role in regulating the initiation, progression, and therapeutic response of diverse malignancies [2]. The TME comprises a complex network of stromal and immune components that dynamically interact with cancer cells, thus shaping tumor behavior [3]. Cancer-associated fibroblasts (CAFs) represent the most abundant and functionally versatile stromal populations in the breast TME [4], where they regulate extracellular matrix (ECM) remodeling, immune responses and tumor cell metabolism. A growing body of evidence indicates that CAFs engage in extensive bidirectional crosstalk with BC cells, influencing their proliferation, invasion and metastatic dissemination [4]. By secreting a variety of soluble factors and remodeling the ECM, CAFs can create a permissive microenvironment that supports tumor progression [5]. Simultaneously, tumor cells can actively reprogram resident fibroblasts into CAFs and modulate their functional state, further reinforcing this dynamic liaison [5]. Distinct CAFs subsets have been associated with diverse biological functions, ranging from matrix production and tissue remodeling to immune regulation and metabolic sustenance [6]. This functional diversity reflects either intrinsic properties of mesenchymal cells or their ability to respond to tumor-derived and microenvironmental cues, highlighting the remarkable plasticity of CAFs within the TME [7].

The study of primary tumor tissue-derived cells has emerged as a powerful approach to investigate tumor biology in physiologically relevant contexts, allowing the preservation of key features of patient-specific tumors [8]. However, the isolation and *in vitro* maintenance of non-immortalized cells remain technically challenging and inherently variable processes. Differences in experimental procedures, including tissue dissociation and cell sorting strategies, culture conditions and media composition, can profoundly influence cell viability, phenotype, and functional behavior [9,10]. Despite their potential impact, such methodological variables are often underappreciated or insufficiently reported, potentially leading to inconsistencies across studies and limiting the reproducibility and comparability of experimental findings [9,10]. In particular, nutrient availability, growth factors, and metabolites within the culture media can significantly influence cellular metabolism, morphology, proliferation rate and transcriptional programs [11]. This may be particularly relevant for CAFs, which display a high degree of plasticity and responsiveness to external signals [12,13]. Consequently, variations in cell culture protocols may induce significant shifts in the functional state of CAFs, including metabolic routes, potentially confounding the interpretation of experimental results and obscuring biologically relevant differences. Importantly, such variability can not only affect baseline cellular phenotypes, but may also influence downstream experimental outcomes, including responses to pharmacological treatments [10,11]. Addressing these limitations is therefore essential to improve the reliability and translational relevance of studies based on tumor tissue-derived cells, including CAFs.

Breast CAFs are usually defined not by a single universal marker, but by a combination of morphology, stromal localization, exclusion of epithelial, endothelial and immune markers, and expression of fibroblast markers such asα-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), platelet-derived growth factor receptor β (PDGFRβ), S100 Calcium Binding Protein A4 (S100A4), among others [4]. Compared with normal mammary fibroblasts, breast CAFs show an activated phenotype, with enhanced ECM deposition and remodeling, contractility, secretion of cytokines and growth factors,–as well as a stronger ability to promote tumor cell proliferation, invasion, immune modulation, angiogenesis and therapy resistance [5]. Despite these differences from normal fibroblasts, many CAFs programs are conserved across tumor types, especially Transforming Growth Factor (TGF)-β-driven myofibroblast activation, ECM remodeling and inflammatory signaling [14]. However, breast CAFs also display tissue- and subtype-specific features related to the mammary microenvironment, including interaction with adipocytes, hormone-dependent programs, and differences between luminal, HER2-positive and triple-negative BC [15]. Thus, breast CAFs share a general CAFs identity with those deriving from pancreatic, colorectal, lung and other solid tumors, even though their transcriptional profile and functional impact are shaped by the tissue of origin and cancer subtype [4]. Accordingly, the isolation of breast CAFs is broadly similar to that used for CAFs from other solid malignancies. Fresh tumor tissue is usually obtained through mechanical and enzymatical digestion to generate a single-cell suspension, or by explant culture, where small tumor fragments are plated and fibroblast-like cells migrate out over several days [16]. CAFs are then expanded and characterized as above described [17,18]. The main differences between the isolation of breast and other tumors may vary since breast tissues often contain abundant adipose components and variable stromal density, which can influence digestion time, tissue handling and fibroblast yield. Highly desmoplastic tumors such as pancreatic cancer may require stronger or longer digestion, while softer or more necrotic tumors may need gentler processing [16]. Matched normal fibroblasts are typically isolated from adjacent non-tumor mammary tissue using the same approaches, allowing direct comparison with tumor-derived CAFs.

In this study, we sought to evaluate the impact of culture conditions on the morphological and functional phenotype as well as the transcriptional identity of CAFs deriving from breast tumors. To this end, we isolated epithelial and stromal cells from female and male BC tissues using a standardized dissociation protocol while applying two distinct culture media throughout both the isolation and maintenance steps. By combining phenotypic characterization with transcriptomic profiling, we aimed to determine whether differences in culture conditions could influence CAFs morphology, growth and gene expression programs. This approach was designed to assess the extent to which methodological variability may affect experimental outcomes and to highlight the importance of standardized conditions when studying primary tumor tissue-derived cells

2. Materials and Methods

Human breast cancer specimens. BC tissues were obtained from female and male patients undergoing surgical resection, following informed consent and in accordance with institutional ethical guidelines and approved protocols, and were processed immediately after collection. Tumor specimens were mechanically minced and enzymatically digested to generate single-cell suspensions as detailed below.

Tissue dissociation and primary cell isolation. Female and male BC samples were processed as previously described [19]. Briefly, tissues were minced into approximately 1–2 mm^3 pieces, washed with phosphate-buffered saline, and placed in Accumax cell detachment solution (Merck, Milan, Italy) for 45 min at room temperature with gentle but constant mixing. After allowing residual tissue fragments to settle (2 min), the supernatant was sequentially filtered through cell strainers with descending pore sizes down to 40 μm to obtain a single-cell suspension. Cells were then diluted either in a baseline-medium named medium A, a Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12—DMEM/F-12 (Life Technologies, Monza, Italy) supplemented with 5% fetal bovine serum (FBS) (Gibco, Life Technologies, Monza, Italy) and 100 μg/mL penicillin/streptomycin (Thermo Fisher Scientific, Monza, Italy), or in a multi-component medium slightly modified from a previously described formulation [20], named medium B. Medium B consists of 1:1 mixture of DMEM and Ham's F-12 (Merck) supplemented with 10 nM triiodothyronine (Merck), 10 mM HEPES, 50 μM ascorbic acid (Merck), 10 nM 17β-estradiol (Merck), 1 μg/mL insulin, 1 μg/mL hydrocortisone, 0.1 mM ethanolamine (Merck), 10 μg/mL transferrin (Merck), 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin (Thermo Fisher Scientific, Monza, Italy), 15 nM sodium selenite (Merck), 2% FBS, 35 μg/mL bovine pituitary extract (Gibco, Life Technologies), and 100 μg/mL gentamicin. This medium was sterilized using a 0.2 μm vacuum filter unit and stored at 4 °C until use. Epidermal growth factor (10 ng/mL) was freshly added immediately before use. Cells were then centrifuged at 240×g for 10 min, and the resulting pellets were resuspended in the respective medium and cultured at 37 °C in a humidified 5% CO_2 atmosphere. After 6 days, fresh medium was added, and within 10–15 days, small cell aggregates (<10 cells) became visible. Adherent outgrowth subsequently generated heterogeneous populations comprising epithelial and mesenchymal-like cells, which were subsequently isolated by fluorescence-activated cell sorting (FACS) to obtain purified cell populations. Specifically, single-cell suspensions were stained with antibodies against epithelial cell adhesion molecule (EPCAM, clone 1B7, 12-9326-42PE, Thermo Fisher Scientific, Monza, Italy) and fibroblast activation protein-α (FAP, FAB3715G, R&D Systems, Bio-Techne, Rome, Italy). Sorted EPCAM$^+$/FAP$^-$ and EPCAM$^-$/ FAP$^+$ cell populations were then maintained and expanded under standard culture conditions.

Cell growth assay. Cells (4×10^4) were seeded in 24-well plates in medium A or medium B, and the proliferation rate was calculated by counting cells every 24 h for 5 days by using the Countess Automated Cell Counter, as recommended by the manufacturer's protocol (Thermo Fisher Scientific, Monza, Italy).

Morphological analysis. Cell morphology was assessed by phase-contrast microscopy at defined time points. Representative images were acquired for each condition. Cell shape was quantified using a polarity index (PI), which is defined as the ratio between the major and minor cellular axes. A PI value close or equal to 1.0 indicates a more symmetric or polygonal morphology, whereas lower values (PI < 1.0) reflect a rounder and less elongated cell shape. Measurements were obtained from multiple random fields across independent experiments.

RNA sequencing. After maintaining CAFs in medium A or medium B for approximately two weeks following tissue dissociation, total RNA was extracted using RNeasy mini kit according to the manufacturer's instructions (Qiagen, Bioset s.r.l., Catanzaro, Italy). RNA integrity for library preparation was determined by analysis of extracted total RNA using a 2100 Bioanalyzer (Agilent Technologies, Milan, Italy) with RNA 6000 NanoChip. RNA concentrations were measured using Qubit RNA Assay Kit. Libraries were prepared from total RNA according to manufacturer instructions with Illumina Stranded mRNA Prep kit. Libraries quality was evaluated by size analysis on 2100 Bioanlyzer (Chip DNA HS, Agilent Technologies, Milan, Italy) and concentrations were determined using Qubit DNA HS assay kit (Thermo Fisher Scientific). Sequencing was performed on Illumina Novaseq X plus in the 150PE format. Reads preprocessing was performed by using fastp v0.20.0 [21] applying specific parameters to remove residual adapter sequences and to keep only high-quality data (qualified_quality_phred = 20, unqualified_percent_limit = 30, average_qual = 25, low_complexity_filter = True, complexity_threshold = 30). The percentage of uniquely mapped reads resulted high with the mean value of 89% (mean value for sample: unmapped reads 6%, quality base > q30 90%). Then, passing filter reads were mapped to the genome reference (Homo sapiens) using STAR v2.7.0 [22] with standard parameters, except for sjdbOverhang option set on read length. Genome and transcript annotations provided as input were downloaded from v105 of the Ensembl repository. Alignments were then elaborated by RSEM v1.3.3 [23], to estimate transcript and gene abundances. Subsequently, the sample-specific gene-level abundances were merged into a single raw expression matrix by applying a dedicated RSEM command (rsem-generate-data-matrix). Genes with at least 10 counts in N samples were then selected, where N corresponds to the sample number in the smallest experimental group. Differential expression was computed by edgeR [24] from raw counts in each comparison. Multiple testing controlling procedure was applied and genes with an FDR ≤ 0.1 and logFC > |0.5| were considered differentially expressed. Annotation of differentially expressed genes (DEGs) was performed using the bioMart package [25] into R 4.3, version 4.4.1 (2024-06-14), querying available Ensembl Gene IDs and retrieving Gene Names and Entrez gene IDs. Principal component analysis (PCA) was performed on RStudio (version 2024.04.2+764) normalized expression data to evaluate sample clustering and variability.

Functional enrichment analysis. Pathway enrichment analysis was performed on DEGs using the Reactome pathway database [26] through the *enrichPathway()* function implemented in the ReactomePA R package (version ReactomePA_1.48.0) [27]. Gene Ontology (GO) enrichment analysis was conducted to identify overrepresented biological processes, molecular functions, and cellular components using the *gseGO()* function implemented in the clusterProfiler R package (version clusterProfiler_4.12.6) [28], based on GO annotations. The following parameters were applied for enrichment analyses: organism = "human" and *p*-value cutoff = 0.05.

3. Results

Distinct culture conditions reshape the phenotypic properties of breast CAFs. The isolation and culture maintenance of primary cancer cells and TME components from patient tissues represents a challenging step that may critically influence downstream experimental outcomes [11]. We isolated BC cells and CAFs from surgically resected tumors of both female and male patients using a combined mechanical and enzymatic dissociation protocol employing two diverse culture media. These media, named medium A and medium B, were used throughout the whole workflow, both for tissue dissociation and subsequent *in vitro* maintenance. Following isolation, epithelial and mesenchymal cell populations were obtained and then purified by FACS analysis on the basis of the expression of the epithelial marker named EPCAM, and FAP that is specifically expressed by activated fibroblasts [19,29]. Considering the key modulatory role played by CAFs within the breast TME [4,30], we focused our downstream analyses on this stromal compartment, also aiming to better capture the high and complex heterogeneity of this population (Figure 1).

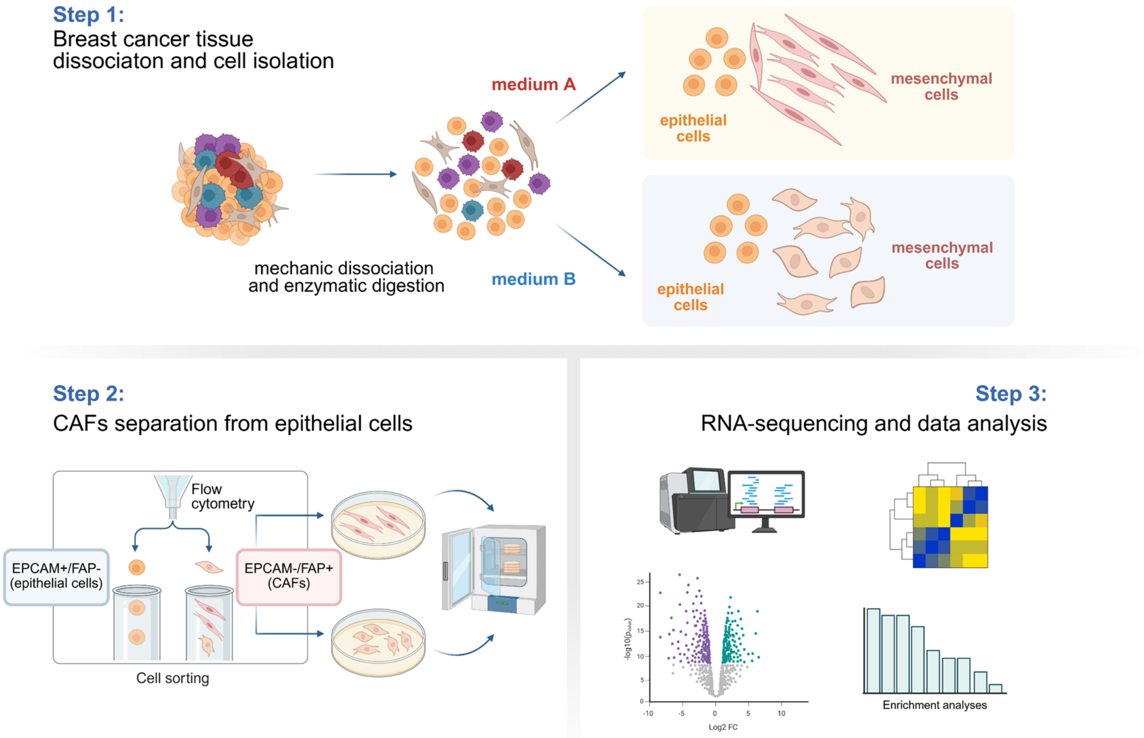

Figure 1. Workflow describing the experimental approach used in the study. Step 1: Female and male breast cancer tissues were mechanically and enzymatically digested in order to obtain a single cell suspension using two diverse culture media, named medium A and medium B. Step 2: Cell suspension was sorted in order to purify epithelial and mesenchymal cells. Step 3: RNA-seq and subsequent bioinformatic analyses were performed to characterize the diverse populations of mesenchymal cells (CAFs) obtained. Created with BioRender.com.

First, the influence of culture conditions on CAFs phenotypic features and behavior was assessed. In this regard, growth kinetics analysis revealed that both female and male-derived CAFs cultured in medium B displayed reduced proliferative rates compared to those maintained in medium A (Figure 2A,B). Consistently, morphological assessment showed that CAFs grown in medium B adopted a markedly less elongated and more rounded morphology respect to those cultured in medium A (Figure 2C,D). Evaluation of cellular shape by quantifying the PI confirmed a significant shift toward lower values in medium B than medium A, which was indicative of reduced cellular elongation in both female and male CAFs (Figure 2E,F). These findings suggest that culture medium composition profoundly affects both the proliferative capacity and morphological state of CAFs *in vitro*.

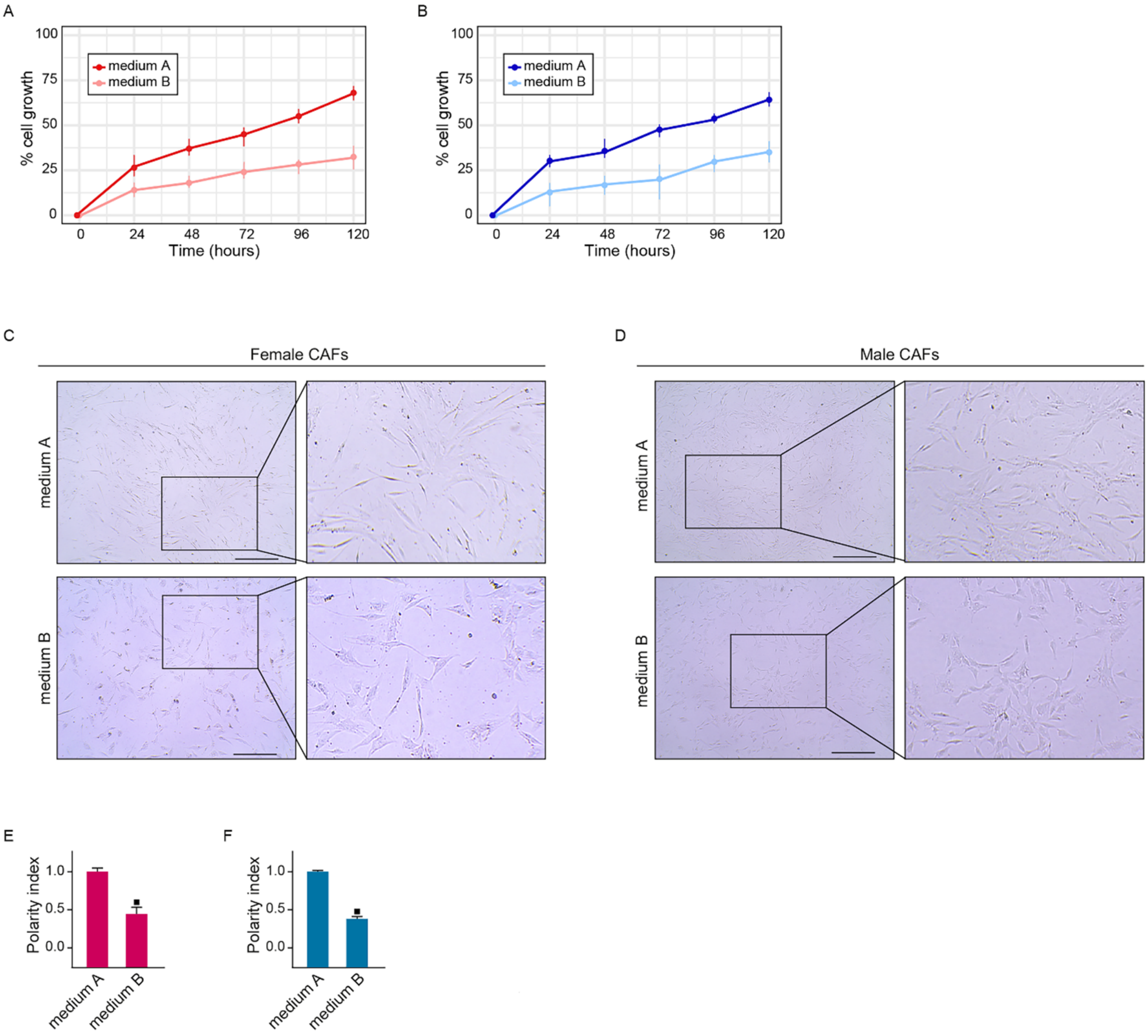

Figure 2. Biological appearance and behavior of female and male CAFs cultured in medium B and medium A. Growth curves of female (**A**) and male (**B**) breast CAFs cultured in medium A or medium B. Morphological appearance of female (**C**) and male (**D**) breast CAFs observed by phase-contrast microscopy. Enlarged details are shown in the side boxes. Polarity index (PI) of female (**E**) and male (**F**) breast CAFs 5 days after seeding and maintained in medium A or medium B. PI = 1.0 indicates a polygonal shape, conversely PI < 1.0 identifies ranges of rounded shapes. The measures shown are representative of 10 random fields acquired in three independent experiments. ■ indicates $p < 0.05$.

Diverse culture conditions in CAFs drive distinct transcriptional programs that are associated with specific biological pathways. Given the extensive heterogeneity of CAFs at both the molecular and functional levels [6,31], comprehensive transcriptomic profiling is essential to capture the diversity of their cellular states and to associate phenotypic traits to the underlying biological behavior. In this context, RNA sequencing (RNA-seq) represents a powerful approach to define comprehensive gene expression patterns, enabling the identification of transcriptional signatures that reflect distinct functional properties and pathway activities [32]. In this regard, to determine whether the observed phenotypic differences were associated with transcriptional reprogramming, we performed RNA-seq on CAFs derived from female and male BC patients cultured in medium A or medium B. PCA revealed clear segregation of samples according to culture condition, indicating that this variable independently contributes to transcriptional variability (Figure 3A). Differential expression analysis identified extensive transcriptional changes induced by culture conditions in both female and male CAFs (Supplementary Tables S1 and S2). As shown in the volcano plots and heat maps in Figure 3B–E, a number of significantly up and down-regulated genes were found in CAFs maintained in medium B compared to medium A (|log2FC| ≥ 1, FDR ≤ 0.01). Collectively, these results indicate that culture medium composition represents a major determinant of CAFs transcriptional identity.

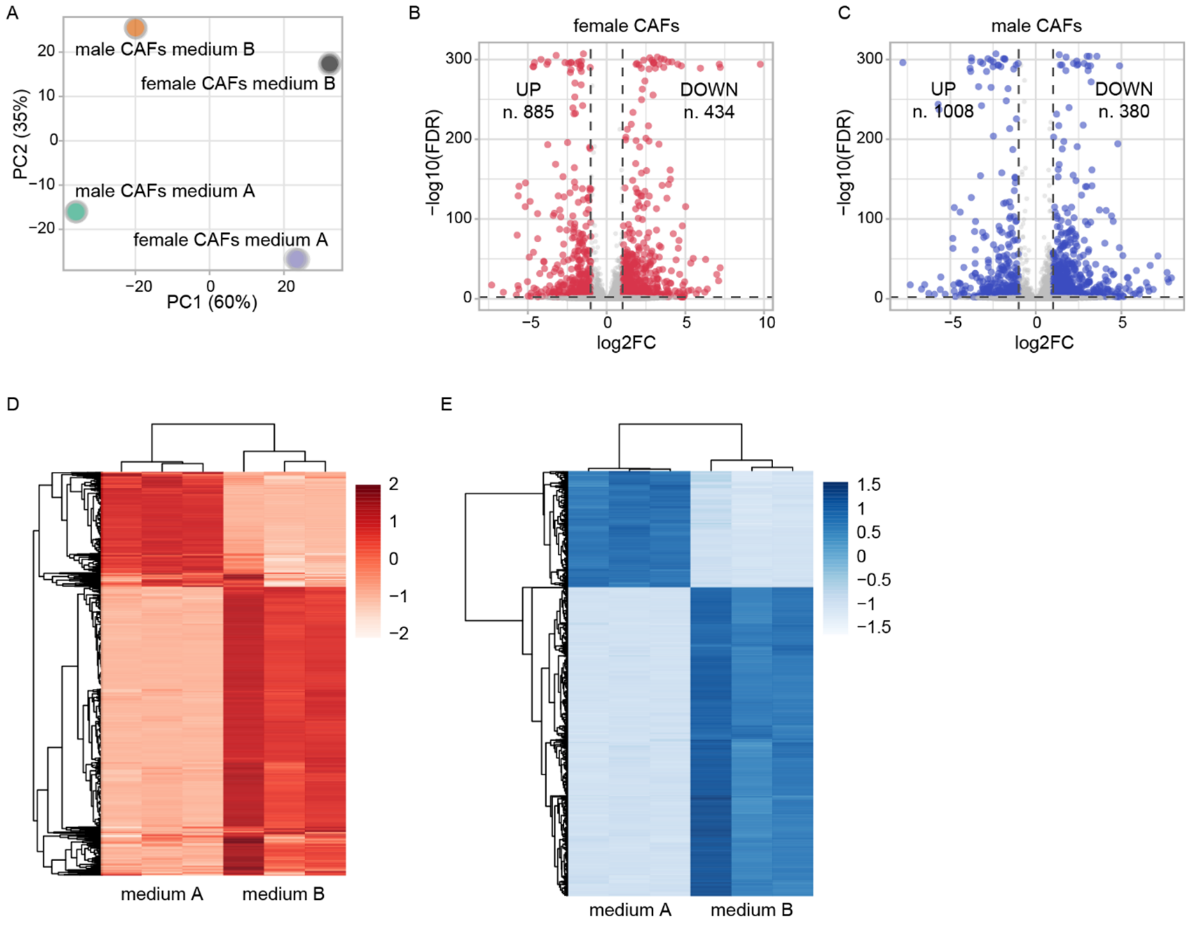

Figure 3. RNA-sequencing (RNA-seq) analysis of female and male CAFs cultured in medium B and medium A. (**A**) Principal component analysis (PCA) plot showing the distribution of female and male CAFs from RNA-seq analysis. Each point represents an individual sample, colored according to group (female or male, medium A or medium B). Volcano plots showing the differentially expressed genes in female (**B**) and male (**C**) breast CAFs maintained in medium B respect to those maintained in medium A. |log2FC| ≥ 1, FDR ≤ 0.01. Heat maps showing the up and down-regulated genes in CAFs isolated from female (**D**) and male (**E**) breast cancer tissues maintained in medium B respect to those maintained in medium A, as identified by RNA-sequencing.

To gain insight into the molecular routes underlying the aforementioned transcriptional changes, we performed Reactome pathway enrichment analysis on the identified DEGs (Supplementary Tables S3–S6). Reactome is a curated pathway database widely used to interpret high-throughput biological data by identifying significantly enriched pathways and inferring their potential activation states from gene expression patterns [26]. In both female and male CAFs, the genes down-regulated in medium B were enriched for pathways involved in ECM organization and remodeling (Figure 4A,C), whereas the up-regulated genes under these conditions were predominantly associated with lipid metabolism-related pathways (Figure 4B,D).

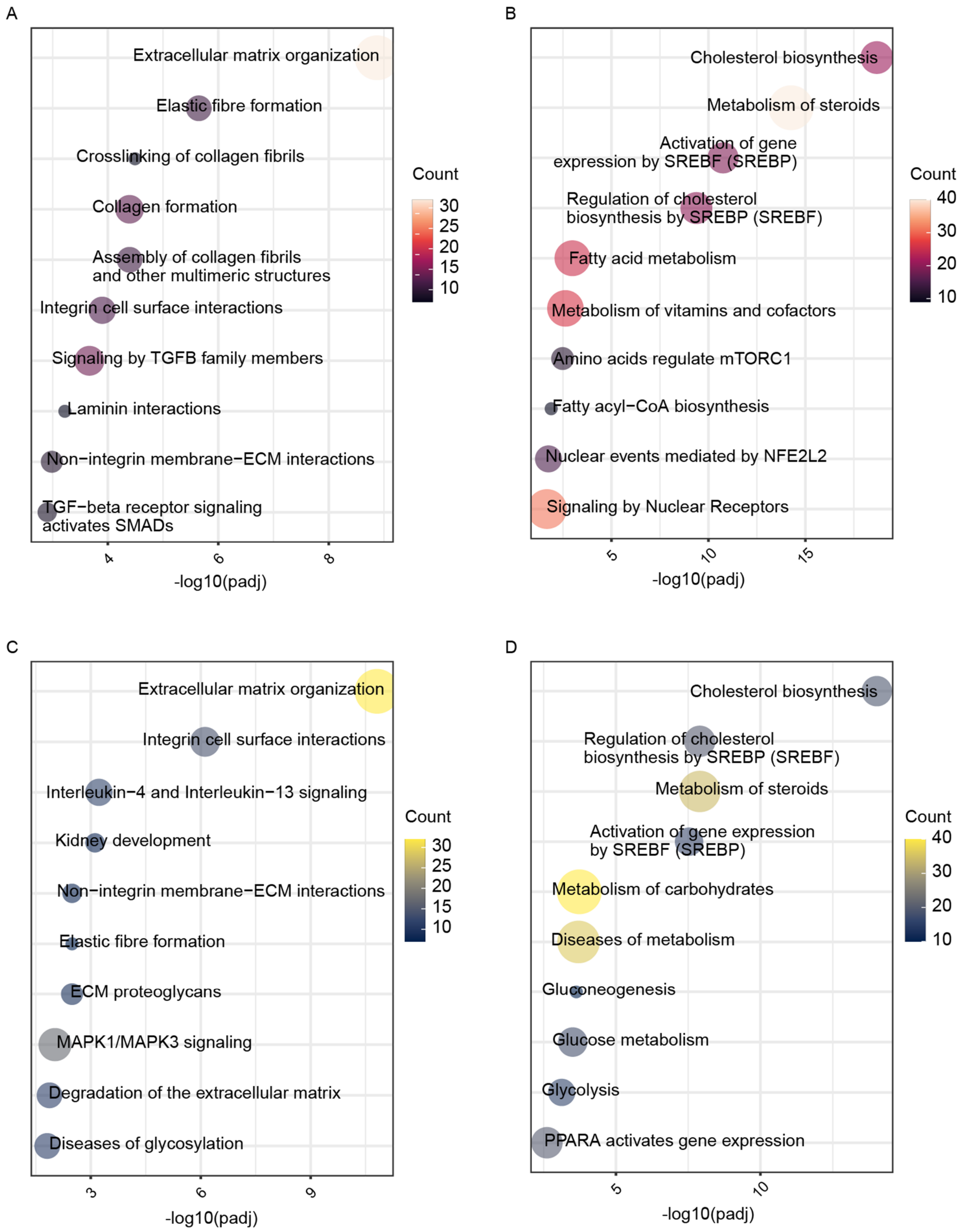

Figure 4. Reactome pathway enrichment analysis of the differentially expressed genes in female and male breast CAFs cultured in medium B respect to those cultured in medium A. Bubble plot showing the top ten enriched pathways for down (**A**) and up-regulated (**B**) genes in female CAFs. Bubble plot showing the top ten enriched pathways for down (**C**) and up-regulated (**D**) genes in male CAFs. Pathways are ranked based on −log10(adjusted *p*-value), as indicated on the x-axis.

GO enrichment analysis, which classifies genes into hierarchical categories based on biological processes, molecular functions, and cellular components [33], further supported these findings (Supplementary Tables S7–S12). In particular, in CAFs cultured in medium B the up-regulated genes were enriched for biological processes linked to metabolic regulation, particularly lipid metabolic processes, while the down-regulated genes were associated with ECM-related functions, including structural organization and cell-matrix interactions (Figure 5A,B). These trends were consistently observed across both molecular function (Figure 5C,D) and cellular component categories (Figure 5E,F).

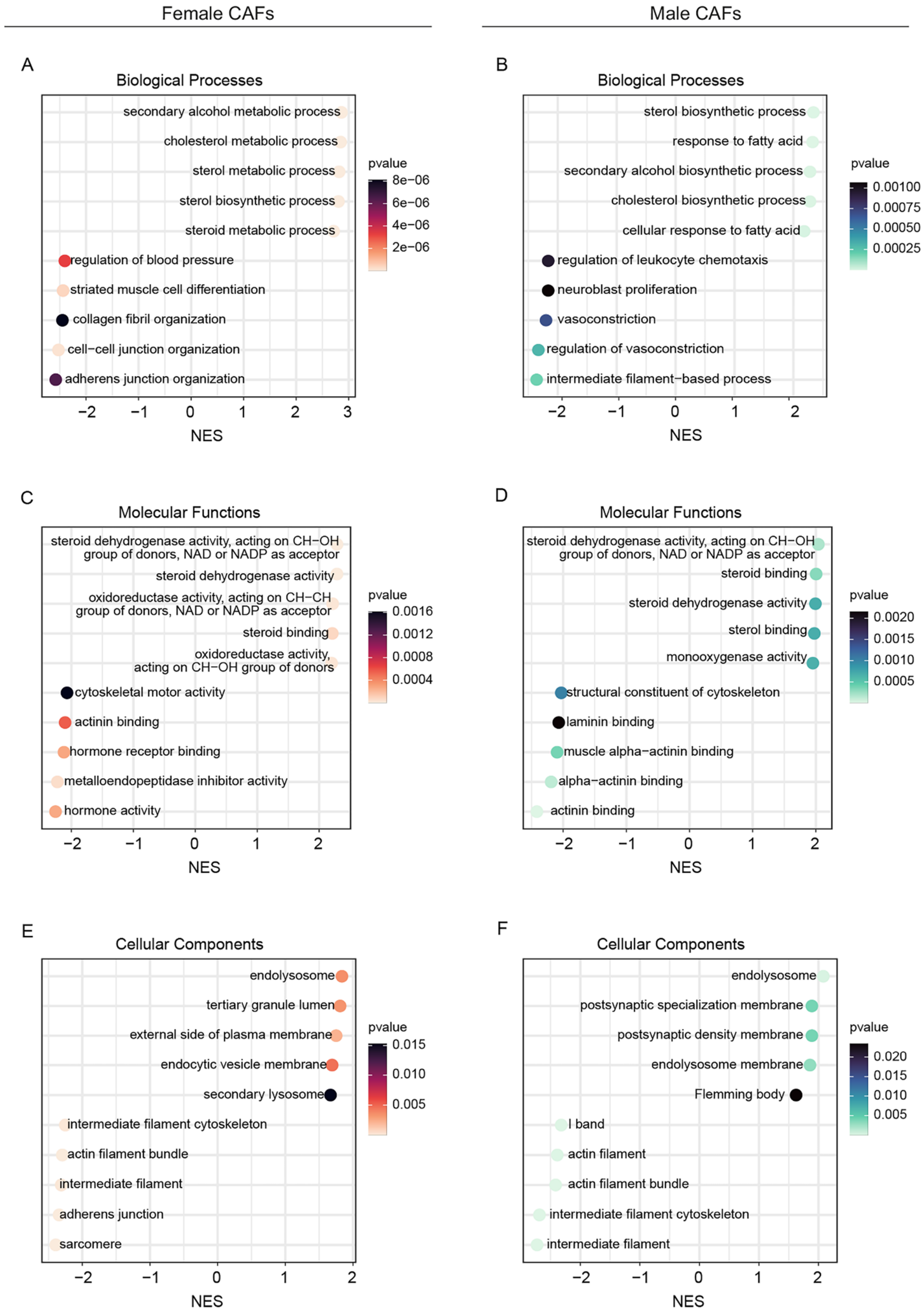

Figure 5. Gene ontology (GO) enrichment analysis of the differentially expressed genes in female and male breast CAFs cultured in medium B versus medium A. Plots depict the GO terms ranked according to the normalized

enrichment scores (NES) and grouped by category: biological processes (**A**,**B**), molecular functions (**C**,**D**), cellular components (**E**,**F**).

Transcriptional signatures shared across female and male CAFs reveal conserved medium-dependent reprogramming. To identify conserved transcriptional responses to culture conditions, we compared the DEGs shared between female and male CAFs. Venn diagram analysis revealed a subset of commonly down and up-regulated genes in female and male CAFs cultured in medium B relative to medium A (Figure 6A,B). Pathway enrichment analysis of these shared genes demonstrated a conserved down-regulation of ECM remodeling pathways, alongside a concomitant increase in lipid metabolism-associated pathways (Figure 6C,D). These findings indicate that culture-induced transcriptional reprogramming of CAFs occurs in a sex-independent manner and converges on key functional axes related to matrix organization and metabolism. Altogether, these data indicate that culture conditions can profoundly influence CAFs phenotype and transcriptional identity, an effect that is consistent across CAFs derived from both female and male BC, and highlight the impact of *in vitro* culture environments on stromal cell identity.

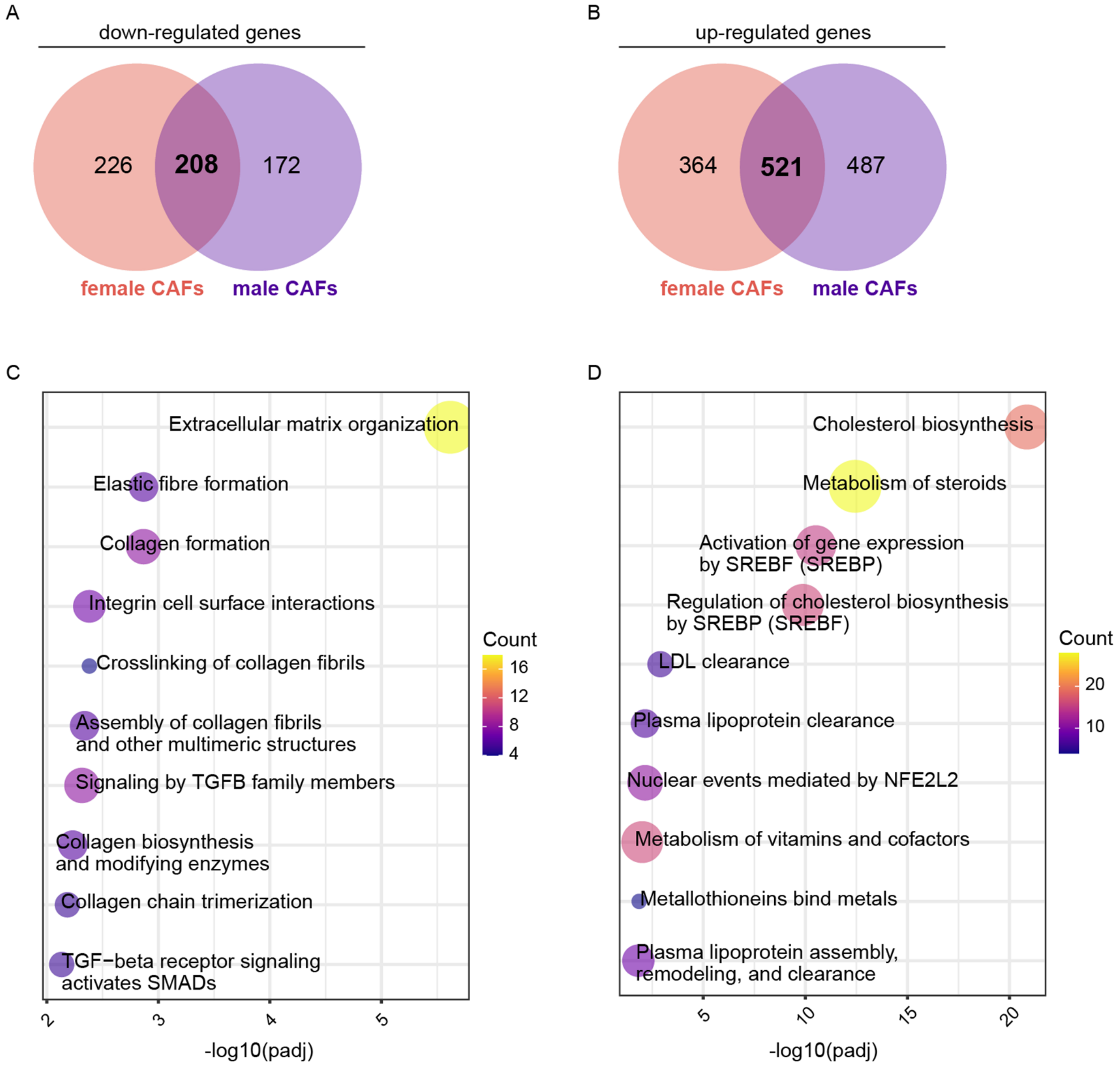

Figure 6. Reactome pathway enrichment analysis of commonly dysregulated genes in female and male breast cancer-associated fibroblasts (CAFs) cultured in medium B versus medium A. Venn diagrams depicting the common down (**A**) and up-regulated (**B**) genes in female and male CAFs cultured in medium B respect to medium A. Bubble plot showing the top ten enriched pathways for the common down (**C**) and up-regulated (**D**) genes in female and male CAFs maintained in medium B respect to those maintained in medium A. Pathways are ranked based on −log10(adjusted *p*-value), as indicated on the x-axis.

4. Discussion

The impact of *in vitro* culture conditions on cellular phenotype and functions remains a critical and underestimated challenge. Despite the extensive reliance on culture models in cancer research, variations in nutrient availability, growth factors and other environmental stimuli can substantially influence cellular metabolism and signaling, ultimately affecting gene expression and experimental outcomes [11,34]. In the present study, we demonstrate that culture conditions may represent a major determinant of CAFs phenotype and transcriptional profile. By systematically comparing two culture media used during both tissue dissociation and *in vitro* maintenance, we found that variations in culture conditions can reshape CAFs morphology, proliferative rate and gene expression programs. These data are in line with previous findings showing that *in vitro* culture conditions can alter cellular phenotypes and metabolic states, thereby impacting experimental outcomes [9,11,35].

The isolation protocol of breast CAFs usually involves the elimination of contaminating cell populations of immune ($CD45^+$), endothelial ($CD31^+$) and epithelial origins ($EPCAM^+$), followed by the validation of canonical fibroblast markers such as α-SMA and FAP [36,37]. In comparison with fibroblasts obtained from non-neoplastic tissues, these cells typically display a more reactive stromal phenotype, including higher matrix-producing activity, increased release of soluble mediators and greater capacity to sustain cancer cell growth, viability and invasion [38–40]. While these functional features are broadly consistent with CAFs populations described in several other malignancies, breast CAFs may also reflect organ-specific influences from the mammary niche, including adipose-rich stroma and hormone-regulated signaling networks. Methods used for their isolation are generally shared across solid tumors, relying on tissue dissociation and subsequent stromal-cell enrichment, with protocol adaptations mainly driven by differences in tissue architecture and fibrosis.

In the present study, cells were maintained under two distinct culture conditions: a baseline medium (medium A) consisting of DMEM/F-12 supplemented with 5% FBS, and a more complex formulation (medium B), based on a 1:1 mixture of DMEM and Ham's F-12 including multiple bioactive supplements such as triiodothyronine, ascorbic acid, 17β-estradiol, insulin, hydrocortisone, ethanolamine, transferrin, sodium selenite, bovine pituitary extract, and 2% FBS. The marked compositional differences between these media are likely to influence cell phenotype and transcriptional programs through the modulation of metabolic activity, growth factor signaling, redox balance, and hormone-responsive pathways. The biological effects of some of these supplements have been previously reported [41–44]. For instance, hydrocortisone has been shown to inhibit collagen production in normal human fibroblasts in the presence of ascorbate [41]. Moreover, fibroblasts derived from patients with homozygous familial hypercholesterolemia has exhibited increased 3-hydroxy-3-methylglutaryl-CoA reductase activity when cultured with triiodothyronine, in accordance with our data indicating an increased expression of its coding gene *HMGCR* in CAFs cultured in medium B [42]. Although the classical estrogen receptor showed low expression in both of our models, 17β-estradiol may affect CAFs behavior thought alternative signaling routes, including the activation of the G-protein coupled estrogen receptor, which is expressed in both female and male CAFs [43,44]. Furthermore, ethanolamine, which is commonly included in defined media as a precursor for phosphatidylethanolamine biosynthesis [45], may support membrane turnover, cell growth and metabolic fitness, although its direct effects on CAFs biology remain to be characterized.

In this complex scenario, our results indicate that CAFs cultured in a medium enriched in hormones, growth factors and supplements display a reduced proliferative capacity and a more rounded morphology compared to those maintained in a baseline medium. These phenotypic changes are accompanied by a marked transcriptional reprogramming, suggesting that culture conditions may influence CAFs toward a specific functional state. This is consistent with the well-established plasticity of CAFs that are cells able to dynamically adapt their molecular phenotype in response to diverse environmental stimuli within the TME [13,46].

At the transcriptional level, we observed a shift in CAFs gene expression based on culture conditions, indicating that media composition is a dominant source of variability. Notably, CAFs maintained in a multi-component medium, which is enriched in hormones, growth factors and supplements, showed the up-regulation of lipid metabolism-related pathways, coupled with the down-regulation of genes driving ECM organization. This shift suggests a transition from a matrix-remodeling phenotype toward a metabolically active state. In this respect, increasing evidence indicates that stromal cells can contribute to tumor growth by providing metabolic substrates to malignant cells, therefore supporting anabolic processes [47,48]. In particular, lipid metabolism has emerged as a key driver of BC progression based on evidence showing that tumor cells can rely on lipid uptake, synthesis and remodeling to sustain their growth and survival [49,50]. In line with these findings, the up-regulation of lipid metabolic pathways observed under diverse culture conditions may reflect an adaptive metabolic state of CAFs that could influence tumor-stroma interactions. Conversely, the down-regulation of ECM-related pathways in CAFs cultured in a supplement-enriched medium suggests an impaired matrix-remodeling capacity, which may have implications for tumor architecture, stiffness and invasion [51]. Additionally, we observed that the culture media-dependent transcriptional changes are conserved across CAFs derived from female and male BC patients, thus supporting that *in vitro* conditions can act as a dominant driver of the behavior of diverse cell types. Consistently, our results suggest that *in vitro* environment can introduce substantial variability in studies employing non-immortalized tumor-derived cell models, based on their capacity to influence baseline cellular states and downstream responses. Accordingly, culture media-driven alterations in metabolic programs and signaling transduction pathways may also influence the sensitivity to pharmacological treatments, potentially leading to discrepancies between *in vitro* and *in vivo* outcomes, as previously reported [10,34,52]. Together, these findings suggest that culture-driven cellular changes may contribute to the limited predictive value of some preclinical models and to challenges in translating experimental observations to the clinic when the complexity of the TME is not adequately represented. These considerations further emphasize the importance of standardized protocols and physiologically relevant culture systems to improve the robustness, reproducibility, and translational relevance of preclinical studies.

Several limitations of this study should be acknowledged. First, although our data provide evidence regarding the impact of culture conditions on CAFs phenotype and transcriptional features, the specific components of the media responsible for these effects remain to be dissected. Future studies aimed at identifying the key metabolic and signaling drivers underlying these changes would provide detailed mechanistic insights. Additionally, extending these observations to more complex co-culture systems would help in determining the extent to which these culture-induced states can reflect or diverge from physiological CAFs behavior.

Moreover, a potential limitation of studies based on cultured CAFs is the loss of native tumor context and spatial interactions. Single-cell RNA sequencing combined with spatial transcriptomics could provide a more accurate *in vivo* characterization of breast CAFs by identifying distinct subpopulations and their localization within the TME, such as invasive fronts, perivascular areas or immune-rich niches [7,53]. These approaches may clarify CAFs functions related to matrix remodeling, immune regulation and therapy resistance [7]. This is particularly relevant in triple-negative BC, where stromal and immune heterogeneity are highly pronounced [54]. Comparative analyses across luminal, HER2-positive and triple-negative tumors may also reveal subtype-specific CAFs programs not retained in culture.

In conclusion, our data show that culture conditions can shape the phenotype and the transcriptional identity of CAFs isolated from both female and male subjects, thus driving relevant metabolic and molecular changes. These findings highlight the importance of carefully considering methodological variables when interpreting results obtained from primary CAF models. For breast CAFs, and more broadly for CAFs derived from other tumor types, we recommend the adoption of setting protocols for tissue processing, cell isolation, medium composition and culture duration. Standardization of these parameters will improve reproducibility across laboratories, facilitate comparison between studies, and enhance the translational relevance of findings generated using patient-derived stromal models.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/2605081125366557/TI-26040019-Supplementary-Materials.xlsx.

Author Contributions

M.T., M.M. and R.L. conceived and designed the study. F.C., D.S., A.Z., M.D.D., A.A.M., F.S., C.M. performed the experiments. M.T., F.C. and D.S. analyzed data. E.M.D.F., R.M., M.M. and R.L. interpreted the results. A.M.M. provided samples. M.T., M.M. and R.L. wrote the manuscript and supervised the research. E.M.D.F., M.M. and R.L. acquired the funding. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

This study was conducted in accordance with the Helsinki Declaration and approved by the Institutional Review Board of the "Comitato Etico Regione Calabria, Cosenza, Italy" (approval code: 500, 25 August 2022).

Informed Consent Statement

Signed informed consent was obtained from all patients.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

Abbreviations

BC, breast cancer; CAFs, cancer-associated fibroblasts; DEGs, differentially expressed genes; ECM, extracellular matrix; EPCAM, epithelial cell adhesion molecule; FACS, fluorescence-activated cell sorting; FAP, fibroblast activation protein-a; FBS, fetal bovine serum; GO, Gene Ontology; PCA, principal component analysis; PI, polarity index; RNA-seq, RNA sequencing; TME, tumor microenvironment.

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