Phenotypic stability of early passage stromal cell lines isolated from prostatic adenocarcinoma

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Abstract

Introduction:

The thorough characterization of markers and phenotypic stability in early passage cell lines is necessary for the proper interpretation of data generated by their use. In our research group, 20 early passage stromal cell lines from prostatic adenocarcinoma (PCa) have recently been isolated. As an initial characterization, the transcript levels of known reactive stromal markers VIM1, TNC and CD90 were measured at three time points to evaluate the phenotypic stability of these novel cell lines.

Method:

One cell line of cancer-associated stromal cells (CAFs) and one of benign prostatic hyperplasia-associated stromal cells (HAFs) from each of 10 patients were seeded in triplicate. RNA was isolated at first confluency from one of the triplicate wells (p0), while the other two wells were harvested at confluency in passages 1 (p1) and 2 (p2). Following cDNA-synthesis, we used reverse transcription real-time polymerase chain reaction to evaluate transcript levels of markers VIM1, TNC and CD90. Intra- and interplate normalization was performed using GAPDH as endogenous control and a calibrator sample included in all plates.

Results:

CD90 was significantly upregulated in CAFs compared with HAFs both at p0 and at p1 and p2. The most sizeable difference was found in the p0 cells. No significant difference was found for VIM1 or TNC. CD90 levels increased with passage, TNC showed no obvious pattern, while VIM1 was stable from p0 to p1, but showed a marked increase at p2.

Discussion:

The higher CD90 expression in CAFs is in accordance with other studies, and indicates that our cells are representative of *in vivo* CAFs. However, the lack of difference between CAFs and HAFs for VIM1 and TNC renders our findings inconclusive. The expression levels of all three markers varied with passage, suggesting a lack of phenotypic stability.

Conclusion:

The data indicate that our cell lines are representative of the *in vivo* situation. However, it would not be advisable to passage them before study, as the differences between CAFs and HAFs appear to decrease over time, and transcript levels vary considerably with passage.

Introduction

In vitro models of human disease are extensively used in the life sciences, and the continuous improvement of methods has done much to bridge the gap between *in vitro* and *in vivo* conditions. Dominating *in vitro* research is monolayer cell culture utilizing commercially available immortalized cell lines. These cell lines have the obvious advantage of being thoroughly characterized, screened for the presence of bacterial, viral and fungal infection, and they can often be passaged indefinitely. While immortalized cell lines permit flexibility and ease for the researcher, it can be challenging to evaluate to which extent these cells are altered from their original *in vivo* phenotype. This uncertainty increases as cells are passaged and subjected to cryopreservation with perhaps multiple freeze/thaw cycles.[1]

The use of primary culture models can in some ways circumvent the challenges of immortalization. In primary culture, cells are cultivated from a tissue of interest, and passaged only until one has achieved sufficient cell numbers for analysis. If several cultures are established from multiple donors, disease heterogeneity can be both studied and taken into account in the interpretation of data. But primary culture is not without challenges. Since every cell line established is unique and of limited availability, results can be difficult to reproduce. As the cells are not immortalized, they will rapidly enter crisis and senescence, drastically changing their phenotype and obliterating the validity of any analysis. [2]

Our research group has previously isolated 20 stromal cell lines using tissue from 10 donors who underwent radical prostatectomy at St. Olavs hospital. However, as each cell line is unique and could react differently to being passaged *in vitro*, it is necessary to characterize them as precisely as possible before subjecting them to further study. A natural starting point for future studies of these cell lines is to utilize high-throughput technologies, such as total-RNA micro-array or RNA sequencing. However, such methods are vulnerable to pre-analytical errors such as the cells themselves not being representative of their origin, or having unstable and non-reproducible gene expression.

A study of gene expression over time will tell us if the cell lines tend to drift phenotypically when passaged. This will have consequences for the design of study models. In addition, by comparing transcript levels in our cell lines with known *in vivo* markers of cancer-associated stromal cells, we can exclude from further study cell lines which have lost their organ- and tissue-specific expression pattern. This will increase the validity of further studies, and hopefully lead to *in vitro* findings more representative of the *in vivo* situation.

We selected three reactive stromal markers for our study. The mesenchymal intermediary filament vimentin (VIM1) is a widely-used marker for cells of mesenchymal origin. It is not entirely specific, inasmuch as it can be expressed in cells of other origins when cultured *in vitro*[3]. However, VIM1 is not only a mesenchymal marker, but its expression has been reported to be higher in the stroma of prostatic adenocarcinoma(PCa) than in that of the normal prostate [4].

An increase of the extra-cellular matrix (ECM) glycoprotein tenascin C (TNC) has also been shown in PCa stroma [4-6]. Our research group has previously failed to reproduce this *in vitro*, and the significance of this marker remains somewhat unclear for the *in vitro* situation.[7]

CD90/THY1 (CD90) is a glycoprotein involved in cell-cell and cell-matrix interactions, and which is expressed in peritumoral stroma. While the transcript has long been perceived as a mesenchymal stem cell marker, recent studies indicate that this is not the case in PCa. Rather, it appears to be a marker of a pro-tumorigenic subpopulation of stromal cells [8].

By measuring the transcript levels of VIM1, TNC and CD90 at three different cell passages for each culture, we aimed to discover more about three aspects of the biology of these early passage cell lines. First, we wanted to characterize the potential differences or similarities of HAFs and CAFs. This will have considerable impact on our strategies for further studying early passage cell cultures from PCa stroma. Second, we wanted an indication of the severity of drift away from the *in vivo* phenotype introduced by the isolation of the cells from tissue and their transfer to in vitro conditions. Finally, we sought new insight into the *in vitro* stability of prostatic stromal cells cultivated by our research group.

Materials and methods

Cell culture and RNA isolation

One early passage culture of hyperplasia-associated stromal cells (HAFs) and one culture of cancer-associated stromal cells (CAFs), isolated from the prostates of each of 10 patients treated with radical prostatectomy at St. Olavs hospital in Trondheim, were included in the study. The patients were between 53-68 years old, and the final Gleason scores ranged from 3+3 to 4+4.

The cell lines had previously been isolated using an explant culture method, and subsequently frozen in 15% DMSO in DMEM/F12 with GlutaMax (Gibco™). For this study, cells were thawed and seeded in triplicate in six-well plates in growth medium consisting of DMEM/F-12 with GlutaMax (Gibco™) with 20% fetal bovine serum and 2% penicillin-streptomycin. The triplicate cultures of each cell line were grown in parallel with medium changes every 3-4 days until near confluency, when RNA from the first well was isolated using the miRNeasy™ Mini Kit (Qiagen™) protocol as per the manufacturer's instructions. These cells were labeled p0, as they were unpassaged after thawing. The two remaining parallel cultures were trypsinized using 0.25 % Trypsin/EDTA and passaged once or twice, respectively, before RNA-isolation at confluency. These cultures were labeled passage 1 (p1) and passage 2 (p2) accordingly. Split ratios were 1:3.

Quality control and dilution

RNA-concentrations were measured using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop products™), and all samples were diluted to 172.5 ng/µL before cDNA-synthesis. A representative selection of 10 samples was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc.) with the Agilent RNA 6000 Nano Kit™ in order to ascertain the RNA-quality.

cDNA synthesis and qRT-PCR

cDNA-synthesis was performed using the SuperScript™ IV VILO™ Master Mix (Invitrogen™) protocol and reagents. Following synthesis, cDNA was diluted 1:10 to provide the sample volume needed for qRT-PCR. qRT-PCR was performed using a StepOnePlus™ Real-Time PCR system, with TaqMan™ probes (Applied Biosystems™) for the transcripts of the genes TNC, VIM1 and CD90 mixed in TaqMan™ Fast Advanced Master Mix (Applied Biosystems™). Gene expression was normalized using a TaqMan™ probe for the housekeeping gene GAPDH. Further, as 8 PCR plates were included in our study, inter-plate normalization was necessary. For this we used a representative sample included in all plates as a calibrator.

Statistical analysis

Gene expression data for the three different cell passages of each culture were compared, and data from the two groups HAFs and CAFs, respectively, were compared. Fold changes were calculated using the ΔΔCt method and Student's t-test in StepOnePlus software v2.3. The same software was used to calculate SEM for the biological groups listed below. Significance threshold was chosen at p=0.05.

Ethics

The Regional Committee for Medical Research Ethics Mid-Norway has approved the use of patient material for this study. All patients included have given an informed, voluntary, explicit and documented consent. Participants can at any time withdraw their donated material from further research use.

Results

Cell culture

Twenty cultures were thawed and seeded in triplicate, a total of 60 cultures, of which 44 displayed successful growth until confluency, and were used for qRT-PCR. It took on average 22.8 days from thawing until confluency and first passage, whereas an average of only 5.5 and 6.6 days, respectively, elapsed between subsequent harvests. Thawing and lysis dates for each culture used for qRT-PCR are shown in Table 1. The RNA quality was assessed in eluates from 10 cultures, of which 9 samples showed a RIN-score of 10, while the last sample was given a score of 9.30.

Table 1: Overview of cell line cultivation and passage dates, including passage intervals in days.

	Patient	Culture	Thawed	P0 lysed	P1 lysed	P2 lysed	Days thaw-P0	Days P0-P1	Days P1-P2
CAFs	4	6	20.10.16	3.11.16	7.11.16	DNRR	14	4	
	5	12	20.10.16	7.11.16	11.11.16	19.11.16	18	4	8
	6	6	11.10.16	3.11.16	7.11.16	11.11.16	23	4	4
	7	11	11.10.16	3.11.16	7.11.16	11.11.16	23	4	4
	9	12	11.10.16	7.11.16	11.11.16	16.11.16	27	4	5
	10	8	20.10.16	3.11.16	DNRR	DNRR	14		
	11	12	20.10.16	16.11.16	30.11.16	DNRR	27	14	
	14	4	11.10.16	3.11.16	7.11.16	11.11.16	23	4	4
HAFs	4	8	20.10.16	31.10.16	3.11.16	11.11.16	11	3	8
	5	9	20.10.16	7.11.16	11.11.16	19.11.16	18	4	8
	6	2	11.10.16	3.11.16	7.11.16	11.11.16	23	4	4
	8	4	11.10.16	3.11.16	7.11.16	14.11.16	23	4	7
	9	8	11.10.16	3.11.16	7.11.16	11.11.16	23	4	4
	10	1	20.10.16	3.11.16	7.11.16	14.11.16	14	4	7
	11	9	20.10.16	7.11.16	11.11.16	16.11.16	18	4	5
	14	10	11.10.16	31.10.16	7.11.16	11.11.16	20	7	4
						Average	22.8	5.5	6.6

Abbreviations: DNRR=Did not reach confluence. DNRR cells were not included in the cDNA synthesis and qRT-PCR.

qRT-PCR

Comparison of the two biological groups HAFs and CAFs

When comparing gene expression of HAFs and CAFs using all 44 samples, we found a significant difference in the transcript levels of CD90, whereas TNC and VIM1 transcript levels were not significantly different between the groups (Fig. 1). CD90 levels were 15% (SD: 1.07-1.23, $p>0.05$) higher in CAFs compared to HAFs.

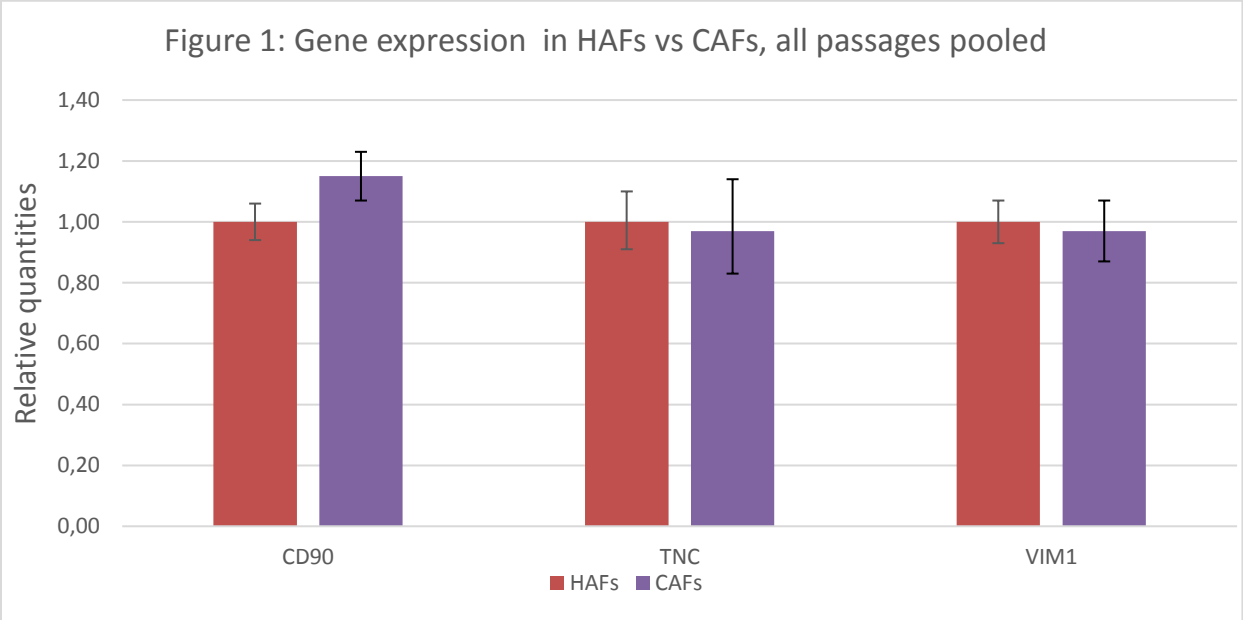

Figure 1: Relative transcript levels in HAFs and CAFs at p0. CD90 displays a significant (p>0.05) difference between the two groups, while differences in TNC and VIM1 levels are non-significant.

However, if we look at the cell cultures in p0 only (Fig. 2), the difference in transcript levels of THY1 is 29% (SD:1.25-1.34, p>0.05).

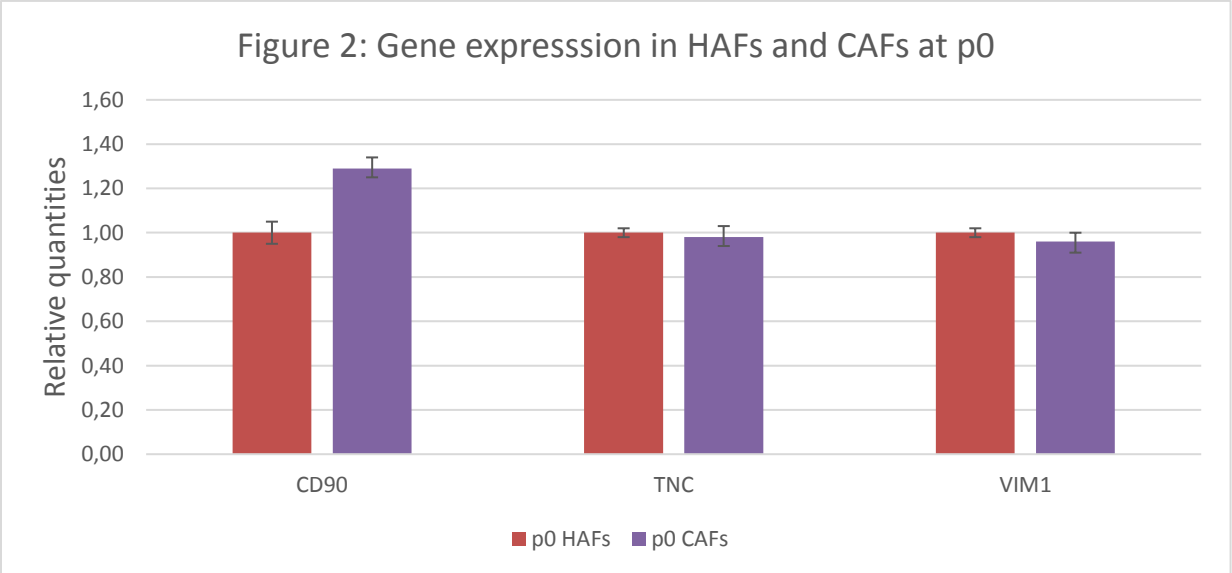

Figure 2: Relative transcript levels in HAFs and CAFs at p0. CD90 displays a significant (p>0.05), and, compared to Fig 1., considerably larger difference between the two groups. Differences in TNC and VIM1 levels remain non-significant.

A more detailed look at the differences in transcript levels can be seen in the following figure, where we find a trend of increasing CD90 expression in both HAF and CAF cultures.

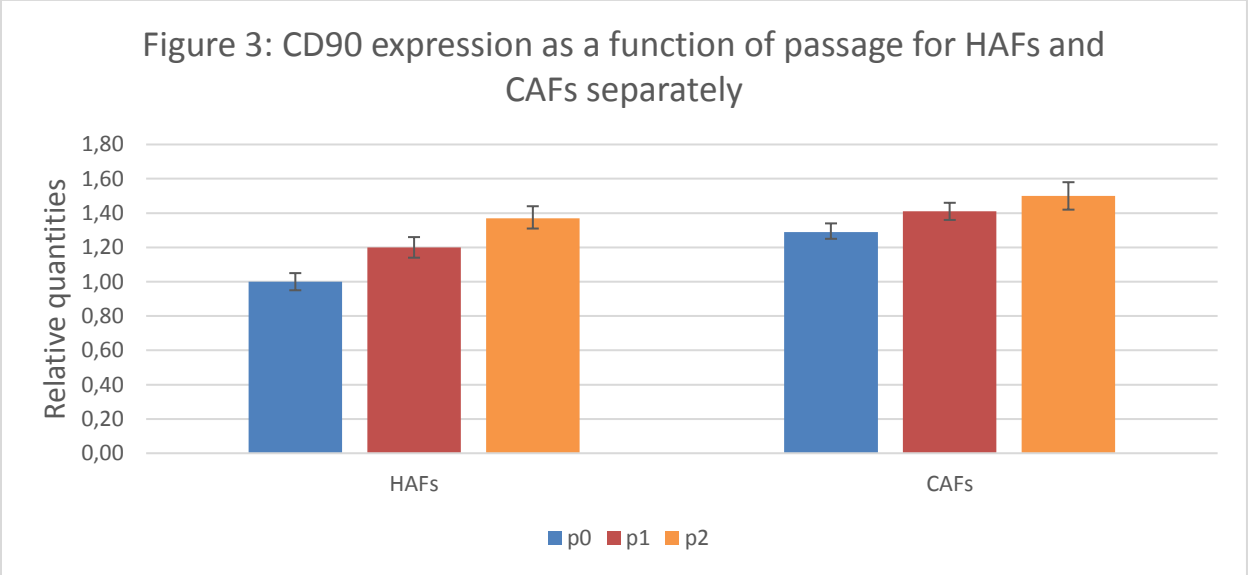

Figure 3: CD90 expression in HAFs and CAFs by passage. In both groups, we see a clear trend of increasing transcript levels with passage. For HAFs all transcript level differences are significant ($p>0.05$), but only the difference between p0 and p1 is significant for CAFs.

Comparison of transcript levels as a function of cell passage

To investigate whether the cultures displayed stable expressions when comparing passages p0-p2, we produced the following figure, showing gene expression as a function of cell passage. There does not appear to be any common pattern in the transcription level changes for the three transcripts investigated. While CD90 displays a marked increase as the cells progress from p0 to p2, TNC shows no consistent pattern, and VIM1 levels appear to be stable from p0 to p1, but are increased in p2.

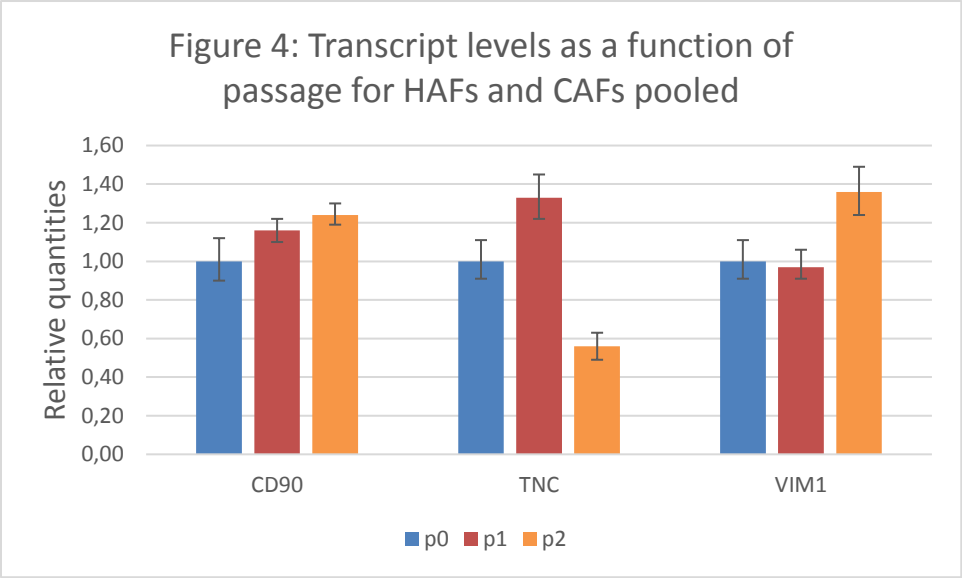

Figure 4: Relative transcript levels for CD90, TNC and VIM1 in p0-p2, HAF and CAF cell lines pooled. No common pattern can be seen for the three transcripts, and only CD90 transcription levels change in a predictable manner.

In Fig 5., we have differentiated between passages and whether the cells are HAFs or CAFs. The variations are quite similar in the HAF and CAF groups, with CD90 expression increasing by passage. TNC expressions are quite varying, and VIM expressions are stable for two passages, before both cell groups markedly increase their expression of the transcript.

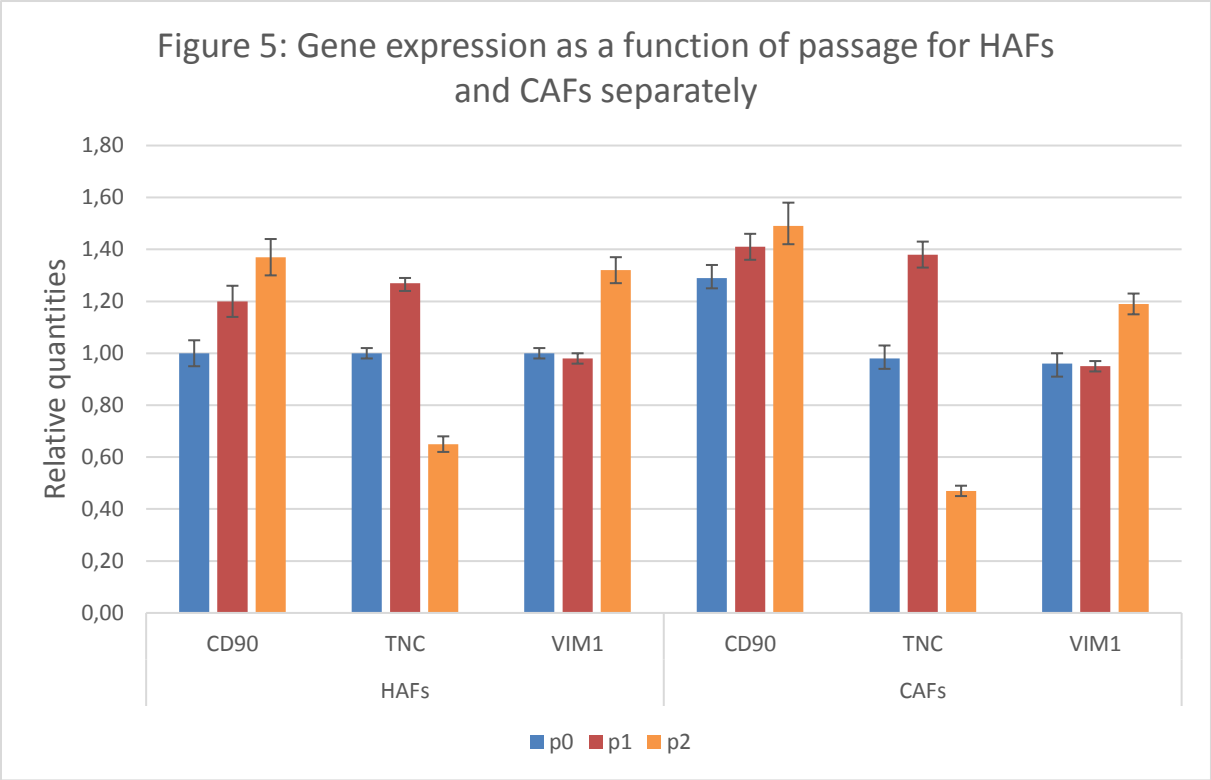

Figure 5: Relative transcript levels for CD90, TNC and VIM1 in p0-p2 for HAF and CAF cell lines separately. HAF and CAF cell lines display almost identical expression patterns for each transcript.

Discussion

Reactive stroma

Studies utilizing immunohistochemistry in the study of prostate cancer tissue have revealed an altered stroma surrounding PCa epithelial cells[4]. In PCa literature, this heterogenous collection of cells in the immediate vicinity of the cancerous cells is often referred to as a reactive stroma. *In vivo* the fibroblasts of reactive stroma express vimentin, tenascin C and secrete increased amounts of pro-collagen 1[9], while myofibroblasts additionally express smooth-muscle actin.

Functionally, reactive stroma is complicit in cancer-promoting ECM remodeling[10] and the epithelial-to-mesenchymal transformation (EMT) necessary for cancer invasion[11]. Cocultivation with reactive stromal cells can even induce tumorigenicity in non-tumorigenic prostatic epithelium [12].

Intimate knowledge of the biology of reactive stromal cells is imperative if we are to understand PCa development and progression. Using explant culture, our research group has attempted to isolate the fibroblasts of reactive stroma, and the cells named CAFs in this study are believed to represent

this cell segment. We performed this study to evaluate whether they express markers typical of other reactive stromal cell lines, and attempted to assess their phenotypic stability over time, as this will influence future study of these early passage cell lines.

Comparison of the two biological groups HAFS and CAFs

In Fig 1., we compare HAFs and CAFs in p0-p2 pooled together, as a first approach to the data. From the figure, we can clearly see that there is no difference between the groups when comparing VIM1 and TNC transcript levels, while there is a significant difference when comparing levels of CD90. However, it is difficult to assess the biological impact of this difference without further investigation. Interestingly, Zhao et al found that only a subpopulation of cultivated stromal cells highly express CD90. They additionally showed that conditioned medium from this highly expressing CD90+ subpopulation (CD90+^{hi}-cells) is able to protect epithelial cells from benign prostatic hyperplasia (BPH) from H_20_2-induced cell death[8].

Thus, the elevated CD90-levels discovered in our CAFs could indicate that a similar subpopulation of CD90+^{hi}-cells exist in our CAF-population. It would be of considerable interest to evaluate if our cells possess the same growth-promoting qualities found by Zhao, and to attempt to elucidate which soluble factors are the main contributors.

VIM1 and TNC levels show non-significant differences between the two groups. This does not model the *in vivo* situation, where several studies have found higher levels of both transcripts in the cells immediately surrounding tumor foci[4, 5]. This illustrates the challenges with *ex vivo* study models, as not all traits of tumor biology are transferrable to the *in vitro* condition. However, this lack of difference could also indicate a selection of cells not representative of the area immediately surrounding the tumor. While this selection is indeed unfortunate, it is difficult to control in an explant culture model.

Impact of cell passage

Fig. 2 shows differences in transcription levels in p0 cells, that have not been passaged after thawing. While VIM1 and TNC levels are still nonsignificant, the difference in THY1-expression is 29%, almost double what is found when comparing all passages combined. It is well known that each passage can be expected to dedifferentiate the cells somewhat, and thus alter the cellular phenotype farther from their origin. However, it is still remarkable that in as little as two passages, differences between the two groups are halved.

Fig 3. is another illustration that cell passage leads to changes in measured transcript levels. We can only speculate if the cause for the gradual CD90-increase with passage is due to a selection of cells similar to $CD90^{hi}$-cells where the selection pressure is stronger in the HAF-group, or if transcription is gradually upregulated in the cell population as a whole as a consequence of *in vitro* cultivation.

While a clear pattern of steadily increasing transcript levels is seen for CD90, no consistent trend can be found for the transcription of VIM1 and TNC. In Fig. 4 we see an increase of TNC levels of 33% from p0 to p1, before levels drop to 56% of P0 levels in P2. VIM1 on the other hand, displays stable levels from p0 to p1, before a dramatic increase at p2.

Remarkably, these patterns are almost identical in HAFs and CAFs, as seen in Fig 5. There is no obvious explanation for these fluctuations, and we find it reasonable to believe that at least the fluctuations for TNC may be due to a random process. It can be argued that VIM1 levels are stable in P0 and P1, but additional passages should have been included in the study to give a definitive answer.

Conclusions

A major objective in this study was to assess whether primary stromal cell lines are stable when passaged *in vitro*. None of the markers included displayed any reassuring degree of stability, although CD90 had a more regular pattern of transcript level changes than the other two markers. The author would thus not recommend that future study models involve passaging of the cells, but rather that they be used as soon as possible after thawing. Alternatively, the use of cocultivation or differentiation through exposure to soluble factors may be necessary to increase the accuracy of future assays.

Additionally, we intended to investigate to which extent the cells were representative of the known *in vivo* reactive stromal population. Based on previous research, CD90 was the marker with the highest biological relevance, as $CD90+^{hi}$-cells are growth promoting for prostatic epithelial cells as found by Zhao et al[8]. A significant and sizeable difference in transcript levels between the CAF and HAF group was indeed found for this marker in P0-cells. For the other two markers, no difference was found. While the CD90-levels increase the likelihood of our cell lines being representative of the *in vivo* situation, our study is rendered at least partially inconclusive by our VIM1 and TNC data, and

additional markers need to be included in the characterization.

Finally, we aimed to establish which, if any, differences exist between HAF and CAF early passage cell lines. The data in this study, although inconclusive on their own, support the existing concept that CD90+-cells are more prevalent in the reactive stroma of PCa. Our study thus indicates that investigating the cells in the CD90+^{hi} reactive stromal compartment may provide functionally relevant and important new clues to tumor-stromal interaction in PCa.

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