TRANSDIFFERENTIATION OF PRIMARY CELLS DERIVED FROM ATHEROSCLEROTIC PLAQUES TO A FOAM CELL LINEAGE

Major research project thesis

Master: Biology of Disease

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ABSTRACT

BACKGROUND: Atherosclerosis is a chronic inflammatory condition which results in the formation of atherosclerotic plaques. Foam cells play an important role in the development of the atherosclerotic plaque by forming the lipid core. It is known that macrophages and smooth muscle cells have the ability to take up oxidised low-density lipoprotein and thereby become foam cells. However, it is not known if transdifferentiated smooth muscle cells in the plaque, also called plaque cells, can also become foam cells via this process.

METHODS: First, the stability of the plaque cells was assessed by generating growth curves and performing qPCR on different passages. Next, plaque cells were exposed to oxidised low-density lipoprotein to check their ability to take up oxidised low-density lipoprotein. Furthermore, different conditions were compared to transdifferentiate plaque cells to the foam cell lineage. Lastly, qPCR was performed on the plaque cells after they were exposed to 50 µg/mL of oxidised low-density lipoprotein for 96 hours in FCS-depletion medium, which were the previously determined conditions.

RESULTS: This study shows that plaque cells have a stable proliferative capacity and stable expression of canonical smooth muscle cell markers until passage 8. Also, plaque cells are capable of taking up oxidised low-density lipoprotein and are able to transdifferentiate to the foam cell lineage when they are exposed to 50 µg/mL of oxLDL for 96 hours in FCS-depletion medium. Furthermore, plaque cells showed no change in smooth muscle cell marker expression and an upregulation of foam cell markers after oxidised low-density lipoprotein exposure.

CONCLUSIONS: These results provide novel insights into the ability of plaque cells to transdifferentiate to a foam cell lineage. Understanding these transdifferentiation capabilities of plaque cells improved the knowledge of the role of smooth muscle cells and foam cells in the development of atherosclerosis.

PLAIN LANGUAGE SUMMARY

Atherosclerosis is a condition in which a plaque is formed in the arteries. An atherosclerotic plaque consists of cholesterol and several cell types, such as macrophages, smooth muscle cells, and foam cells. If the atherosclerotic plaque ruptures, the content of the plaque comes in contact with the blood in the bloodstream. This results in a blockage of the artery and elicits myocardial infarction and brain infarction which are life-threatening (1).

One of the cell types present in the atherosclerotic plaque which plays an important role is the foam cell. These foam cells are filled with a type of cholesterol, oxidised low-density lipoprotein (oxLDL), and are present in the core of the plaque. It was long thought that the only cell type able to take up the oxLDL in the plaque were macrophages. When the macrophages are filled with oxLDL, they are called foam cells. Since a few years, it is known that smooth muscle cells are also capable of taking up the oxLDL and transforming into foam cells (2).

In healthy conditions, smooth muscle cells are present in the arterial wall. However, during atherosclerosis some smooth muscle cells detach from the arterial wall and infiltrate the plaque. These smooth muscle cells have different characteristics compared to the smooth muscle cells present in the arterial wall (3). When they are present in the atherosclerotic plaque, they are called plaque cells. Considering these plaque cells are of smooth muscle cell origin, the question arose if these plaque cells were also able to take up oxLDL in the plaque and become foam cells. The aim of this study was to answer this question.

This study found that plaque cells are stable during culturing in the laboratory and therefore are appropriate to use in experiments. Furthermore, plaque cells indeed are able to take up the oxLDL. When the plaque cells were exposed to oxLDL with a concentration of 50 µg/mL for 96 hours, the cells switched to foam cells. This was based on the fact that the plaque cells switched in shape and became more like a foam cell. Besides looking at the shape of the cells, their gene expression was also checked. A selection of genes which are specific for smooth muscle cells and genes specific for foam cells were used to check the expression levels. The expression of these selected genes was compared between the plaque cells before and after exposure to oxLDL. The expression of smooth muscle cell-specific genes did not change, while the expression of the foam cell-specific genes increased after exposing the cells to oxLDL. This means that the plaque cells both externally and internally switched to foam-like cells as seen in their change of shape and gene expression, respectively.

The results from this study improved the knowledge of the role of smooth muscle cells, plaque cells, and foam cells in atherosclerosis.

LIST OF ABBREVIATIONS

CEA	carotid endarterectomy
EC	endothelial cell
ECM	extracellular matrix
HCASMC	human coronary artery smooth muscle cell
MSC	mesenchymal stem cells
oxLDL	oxidised low-density lipoprotein
TEA	tibial endarterectomy
VSMC	vascular smooth muscle cell

1. INTRODUCTION

Atherosclerosis is a chronic inflammatory condition affecting the tunica media and intima of large arteries leading to the formation of plaques through a process called atherogenesis (1,4). This process involves several cells types, including endothelial cells (ECs), vascular smooth muscle cells (VSMCs), macrophages, foam cells, other inflammatory and dendritic cells, and different molecular mechanisms such as endoMT, and VSMC phenotypic switching (1,4–6). Clinically, atherosclerotic plaques might remain asymptomatic for years before leading to cardiovascular events such as myocardial infarction, angina pectoris, heart failure, or stroke (4). These clinical manifestations arise via two mechanisms known as plaque rupture and superficial erosion. Plaques prone to rupture are defined as unstable while the ones undergoing erosion are defined as stable. The unstable phenotype consists of a large lipidic/necrotic core, a small amount of matrix, a small number of VSMCs, and a high number of macrophages and foam cells. While the stable phenotype consists of a small lipidic/necrotic core, a large amount of matrix, a high number of VSMCs, and a small number of macrophages and foam cells (7–9). All things considered, one of the macro differences between the plaque phenotypes is the presence or absence of a lipidic core, which is mainly formed by foam cells. The excessive influx of oxidised low-density lipoprotein (oxLDL) in combination with the accumulation of cholesterol esters during the process of atherosclerosis, results in the formation of foam cells (2). Those cells have been thought to only originate from macrophages. However, recently a lot has been discovered about their SMC derivation (4,10–14). Several studies have shown that at least 50% of the foam cell fraction in atherosclerotic plaques is of transdifferentiated VSMCs origin as a result of their oxLDL uptake (15–17).

VSMCs are known to retain a certain degree of plasticity which makes them capable of switching toward other cell lineages (Figure 1)(4). Upon certain stimuli, medial VSMCs lose myocardin expression, detach from the tunica intima, and migrate toward the endothelium. Afterwards, these modulated VSMCs undergo a KLF4-dependent process giving rise to VSMC-derived plaque cells. These cells might have high plasticity and therefore are capable of switching toward other cell lineages involved in plaque progression, such as foam cells, macrophages, myofibroblasts, mesenchymal stem cells (MSC), etc (3,4,18). This transdifferentiation of SMC-derived cells results in a decrease of VSMC markers, such as S100A4, PDGFβ, KLF4, ACTA2, MYOCD, and TAGLN. Therefore, in cells derived from SMCs, such as foam cells, VSMC markers are downregulated while foam cell markers (CD64, CD68, CD86, CD163, ABCA1, LGALS3, PLIN2, CD44) are upregulated (2).

As mentioned, VSMCs need certain stimuli to be able to gain transdifferentiation capabilities and for example eventually switch to other cell lineages. Newman *et al.* have shown that VSMCs shift to a more active state after treating them with PDGFβ (19). Consequently, these activated VSMCs were more prone to transdifferentiate toward other cell lineages when exposed to different triggers. Besides, Sakic *et al.* observed a similar effect after stimulation of porcine and murine VSMCs with PDGFβ and oligomeric S100A4 (20). They found a morphological change, activation of pro-inflammatory pathways (NF-κB), and increased proliferative capacities in stimulated VSMCs.

Considering the stimuli through which VSMCs get modulated and transdifferentiate toward other lineages in the atherosclerotic lesions, Buono *et al.* isolated and cultured VSMC-derived cells directly from atherosclerotic lesions obtained from patients who underwent carotid endarterectomy (CEA) or tibial endarterectomy (TEA)(21). These primary VSMC-derived cells, defined as plaque cells, have been characterized and were shown to have SMC derivation, resemble plaque SMCs, and retain patient gene expression. Additionally, they are very active in migration, proliferation and in the production of extracellular matrix (ECM) components (22,23).

In this study, the hypothesis is that plaque cells are capable of taking up oxLDL and transdifferentiating toward a foam cell lineage. That is why understanding how plaque cells transdifferentiate toward foam cells would offer a great opportunity to increase knowledge of plaque progression mechanisms and would aid in the search for novel patient-specific therapeutic targets.

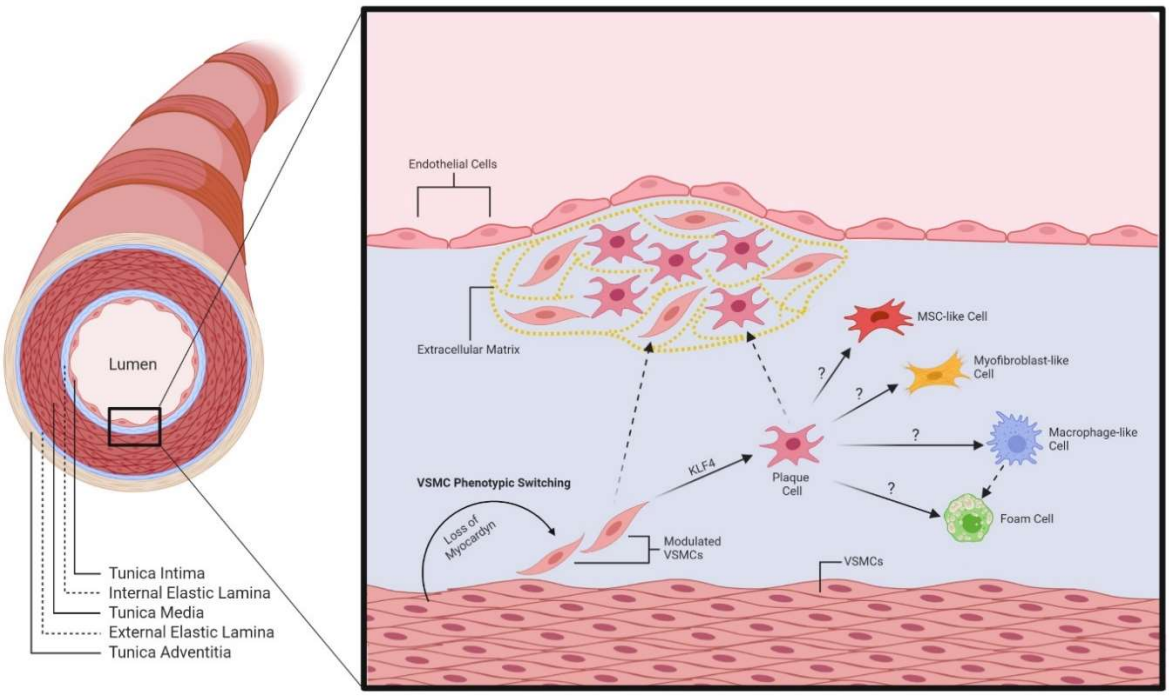

Figure 1: Plasticity of VSMCs in the atherosclerotic plaque. Upon certain stimuli, medial VSMCs lose myocardin expression, detach from the tunica intima, and migrate toward the endothelium. Afterwards, these modulated VSMCs undergo a KLF4-dependent process giving rise to VSMC-derived plaque cells. These cells might have high plasticity and therefore are capable of transdifferentiating toward other cell lineages involved in plaque progression, such as foam cells, macrophages, myofibroblasts, mesenchymal stem cells, etc. (VSMC, vascular smooth muscle cell; MSC, mesenchymal stem cell). Adapted from Buono *et al.* **(3)**.

2. Results

2.1. Isolated plaque cells maintain a constant growing rate for 8 passages

To understand for how long the plaque cells could be kept in culture and reliably used for experiments, primary plaque cells from six donors have been used to generate growth curves. These showed that plaque cells have an average doubling rate of approximately four days until they reach passage 8. After that, the proliferative capacity decreases (Figure 2A) and the plaque cells are not usable for experiments anymore. Sex-stratified growth curves have been also generated (Figure 2B) showing no differences between the female and male donors. In comparison, commercially available HCASMCs have shown greater survival by continuing to grow steadily up to passage 11.

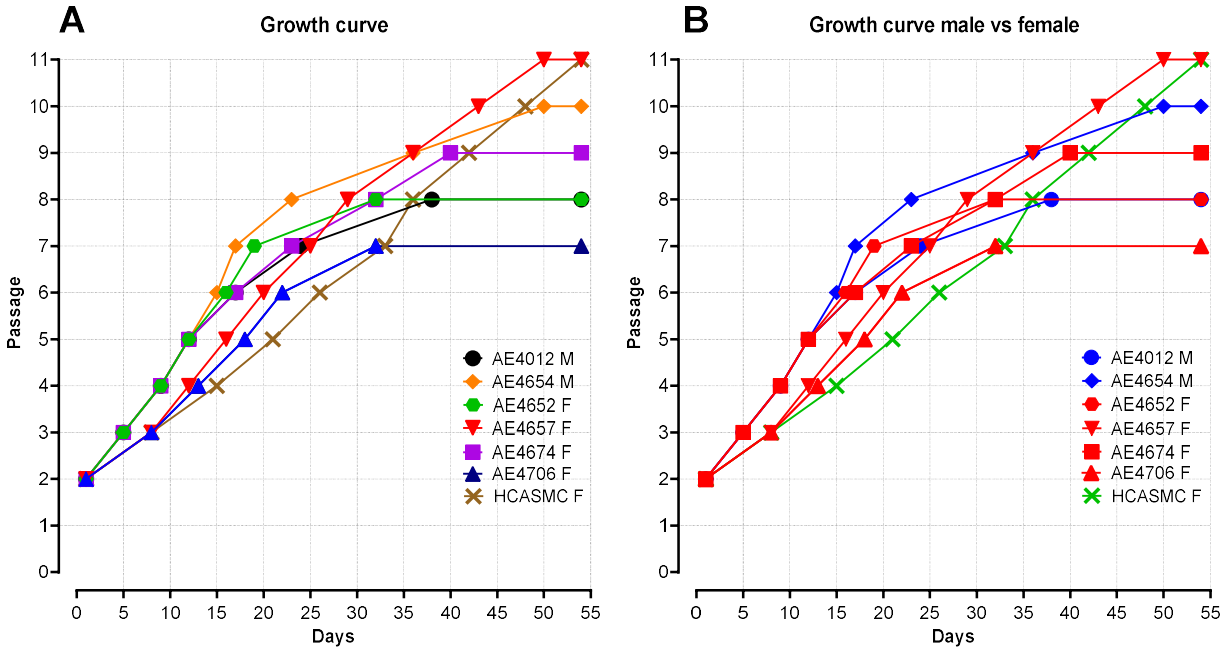

Figure 2: Growth curve of plaque cells and HCASMCs. **(A)** Growth curve to compare all the cell lines. **(B)** Growth curve to compare male (blue) and female (red) cell lines. (HCASMC, human coronary artery smooth muscle cell)

2.2. CANONICAL SMC MARKERS ARE STABLY EXPRESSED OVER TIME IN PLAQUE CELLS

Given the SMC-derivation of plaque cells, the expression of canonical SMC markers, such as ACTA2, MYOCD, TAGLN, CNN1, CALD1, MYH11, MYH10, PDGFβ, KLF4, TPM4, and MMP2, has been checked in plaque cells and HCASMCs during prolonged cultures. Total RNA has been isolated from passage 2 to passage 11 and qPCR has been performed to check the change in the expression of SMC markers over time. Overall, plaque cells have shown stable expression of canonical SMC markers over passage 2 to 11 (Figure 3A). Conversely, not all SMC markers show a completely stable expression over time in plaque cells. Of interest are CALD1, MYH10, PDGFβ, and KLF4, because some passages show a significant fold change compared to passage 2. However, the observed significant fold changes are small.

On the other hand, higher expression variability over the passages for canonical SMC markers in HCASMCs was observed (Figure 3B). MYH11 got upregulated by 60 folds, while PDGFβ showed 12 folds upregulation over culture. These findings made us conclude that plaque cells are overall genetically stable, while HCASMCs show more variability in the over time expression of canonical SMC markers.

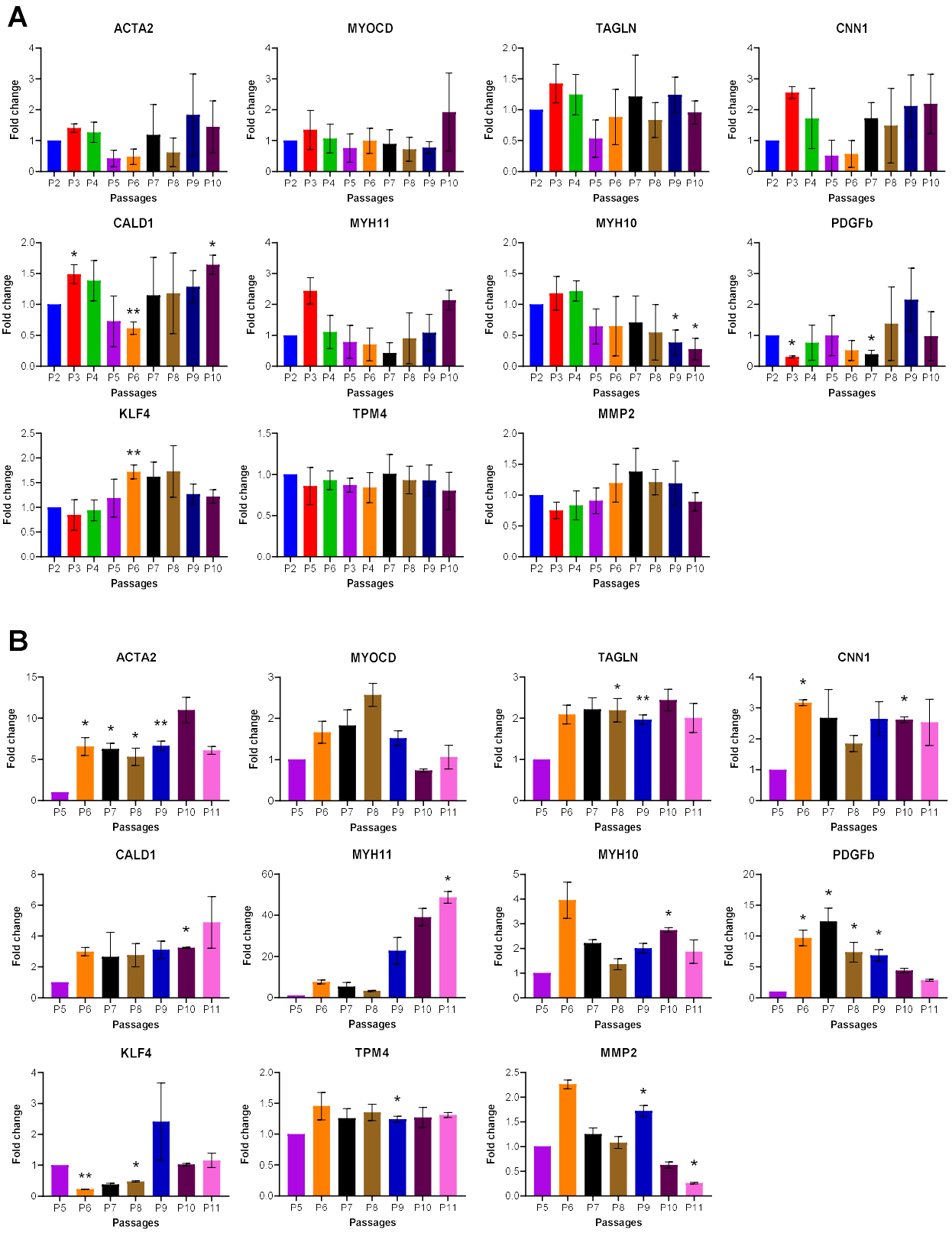

Figure 3: Over time expression of canonical SMC markers in HCASMCs and plaque cells over passage 2 to 11. **(A)** Gene expression of plaque cells over passage 2 to 10, compared to passage 2. Average from 5 different plaque cell lines (N=5) ± SD. **(B)** Gene expression of HCASMCs over passage 5 to 11, compared to passage 5. Average from 2 cell lines (N=2) ± SD. Comparisons were performed by using unpaired t-test. Ns $p>0.05$, * $p<0.05$, ** $p<0.01$. (HCASMC, human coronary artery smooth muscle cell)

2.3. PLAQUE CELLS ARE CAPABLE OF TAKING UP OXLDL

In atherosclerotic plaques, macrophages and modulated VSMCs have been shown to be capable of transdifferentiating toward the foam cell lineage after an excessive influx of oxLDL in combination with accumulation of cholesterol esters (2). Given the SMC-derivation of plaque cells, it was tested whether they were capable of uptaking oxLDL and eventually transdifferentiating to a foam cell lineage.

First, plaque cells have been exposed to different concentrations of pH-sensitive conjugated oxLDL (2.5; 5.0; 12.5; 25 µg/ml) and observed at different timepoints (6, 12, 24, 48, 72, 96h). Then, fluorescent images were taken from two different patients which showed that plaque cells were capable of taking up oxLDL (Figure S1). The images show blue dots and red dots which represent the nuclei and taken up oxLDL, respectively. Moreover, it could be concluded that oxLDL uptake by plaque cells is dependent on oxLDL concentration and time.

Furthermore, the oxLDL uptake from the fluorescent images was quantified by using a macro protocol in ImageJ (Figure 4). The figure shows the relative oxLDL uptake normalized per cell for the different oxLDL concentrations and incubation times. Based on this figure, it could be concluded that both a longer incubation time and a higher oxLDL concentration result in an increase in lipid uptake per cell compared to the control. The first significant increase in lipid uptake is already present after 6 hours of incubation.

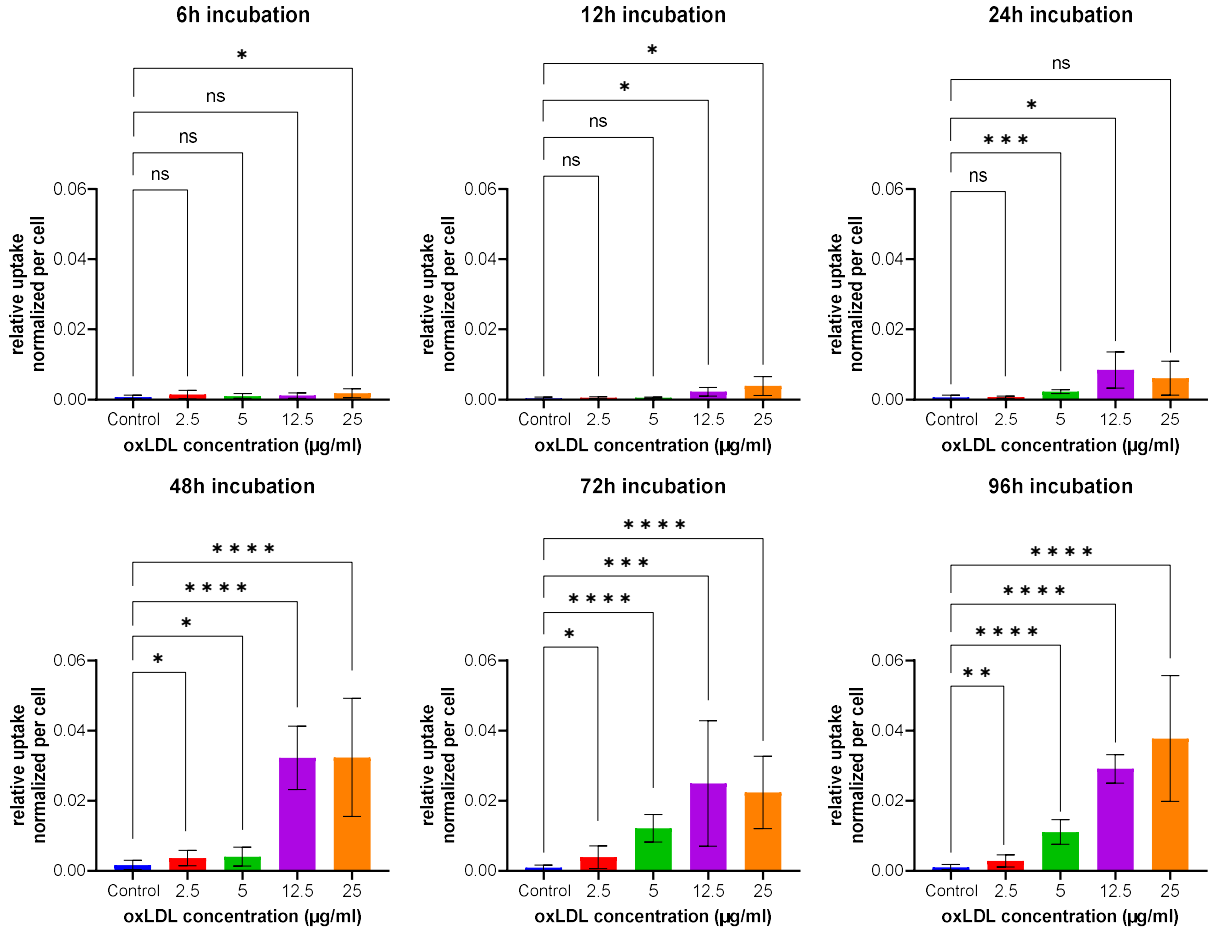

Figure 4: Relative oxLDL uptake in plaque cells normalized per cell. Different incubation times (6, 12, 24, 48, 72, 96 hours) and oxLDL concentrations (control; 2.5; 5.0; 12.5; 25 µg/ml) were analysed. Average relative uptake normalized per cell ± SD is shown and compared to the control condition. Plaque cells from 2 different patients (N=2) were used to acquire these results. Ns $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Comparisons were performed by unpaired t-test. (oxLDL, oxidated low-density lipoprotein)

2.4. PLAQUE CELLS TRANSDIFFERENTIATE TO A FOAM CELL LINEAGE

Given the capability of plaque cells to take up oxLDL, different concentrations of oxLDL, culture media, and incubation times have been used to push plaque cells to transdifferentiate toward the foam cell lineage. Besides, based on the studies of Newman *et al.* and Sakic *et al.* two different molecules, which are known to enhance the transdifferentiation capabilities of SMCs, have been taken along (19,20). Plaque cells from two different patients and HCASMCs have been stimulated with PDGFβ, S100A4, or a combination of PDGFβ and S100A4 for 4 days in complete medium. After that, the cells have been exposed to different oxLDL concentrations (12.5, 25, 50 µg/ml) for several incubation times (24, 48, 72, 96, 120 hours) in either starvation or FCS-depletion medium. Transmitted light images have been taken of the different conditions and were used to compare and select the optimal transdifferentiating conditions (Figure S2-S5).

The effects of the different stimulants are shown in Figure S2. In both plaque cells and HCASMCs the preferred method is no stimulation, which means only exposure to oxLDL. This is based on the fact that the addition of stimulation had no extra effect on phenotypic switching. The cells did not show more frequent or more clear phenotypic switching after the cells were stimulated.

Secondly, the different media types could be compared which included FCS-depletion and starvation medium. The cells showed a clear phenotypic switching in FCS-depletion medium, while the cells were dying in starvation medium. Thirdly, three different oxLDL concentrations were compared. Both the plaque cells and HCASMCs showed the clearest phenotypic switching when exposed to 50 µg/ml of oxLDL. Lastly, the incubation time also influenced the transdifferentiation process. Plaque cells were starting to transdifferentiate after 72 hours with a peak after 96 hours of incubation. After 120 hours of exposure to oxLDL, the plaque cells were dying. HCASMCs, on the other hand, showed a little change in morphology after 24 hours of incubation by becoming less spindle-shaped than normal. However, after 24 hours no further changes in their phenotype had been observed.

To summarize, comparing the medium types, oxLDL concentrations, and incubation times resulted in the following conclusions. Plaque cells from patient AE4674 had the clearest phenotypic switching in FCS-depletion, with 50 µg/ml oxLDL, and incubated for 96 hours (Figure S3). Plaque cells from patient AE4657 did not show phenotypic switching or any relevant morphological change (Figure S4). Lastly, the HCASMCs changed the most evident in FCS-depletion medium, with 50 µg/ml oxLDL, and incubated for 24 hours (Figure S5).

Based on these above-reported results, the optimal conditions to push plaque cells to transdifferentiate toward the foam cell lineage have been established as no stimulation, in FCS-depletion medium, with 50 µg/ml of oxLDL, and incubated for 96 hours. To confirm these findings plaque cells from patient AE4808 were also exposed to the determined conditions. These cells showed phenotypic switching after 96 hours of incubation, like the plaque cells from patient AE4674 (Figure 5). This figure also shows the difference between the plaque cells and the HCASMCs in how and when they change after oxLDL exposure.

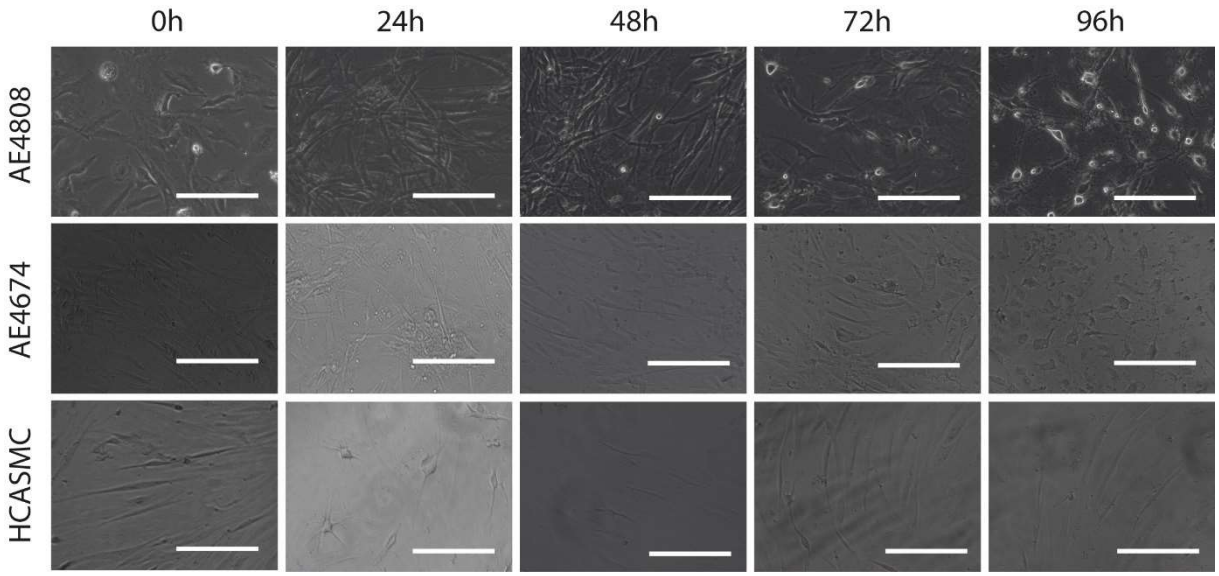

Figure 5: Transmitted light images of plaque cells from patient AE4808, and AE4674, and HCASMCs. The cells were incubated with 50 µg/ml of oxLDL in FCS-starvation medium for 5 different timepoints (0, 24, 48, 72, and 96h). Bar = 200 µm. (FCS, fetal calf serum; oxLDL, oxidated low-density lipoprotein; HCASMC, human coronary artery smooth muscle cell)

2.5. UPREGULATION OF FOAM CELL MARKERS AFTER OXLDL EXPOSURE

The previously established optimal conditions were used to collect the total RNA of plaque cells and HCASMCs, before and after oxLDL exposure, to determine the normalized gene expression of VSMC (S100A4, PDGFβ, KLF4, ACTA2, MYOCD, TAGLN) and foam cell (CD64, CD68, CD86, CD163, ABCA1, LGALS3, PLIN2, CD44) markers.

Figure 6 shows the normalized gene expression of the VSMC markers in plaque cells and HCASMCs. Overall, the VSMC marker genes are not changed after exposure to oxLDL. However, some comparisons between the control and oxLDL condition are significant. It is of note that most significant changes are found in HCASMCs and not in plaque cells. The only markers with significant changes in plaque cells are MYOCD and KLF4. MYOCD shows a significant increase in expression after 96 hours of exposure to oxLDL. Furthermore, KLF4 shows a small but significant decrease in expression after 48 of incubation with oxLDL.

On the other hand, the foam cell markers are significantly upregulated in the oxLDL condition compared to the control condition in both plaque cells and HCASMCs (Figure 7). All foam cell markers follow a pattern of increased gene expression compared to the control condition and the biggest change is seen after the longest incubation time. A gene of note is CD86, which shows an outstanding difference between HCASMCs and plaque cells with a downregulation and upregulation, respectively. Another striking fold change is found in the expression of PLIN2. It is significantly upregulated in all conditions in both cell types and has a big change in expression compared to the other genes.

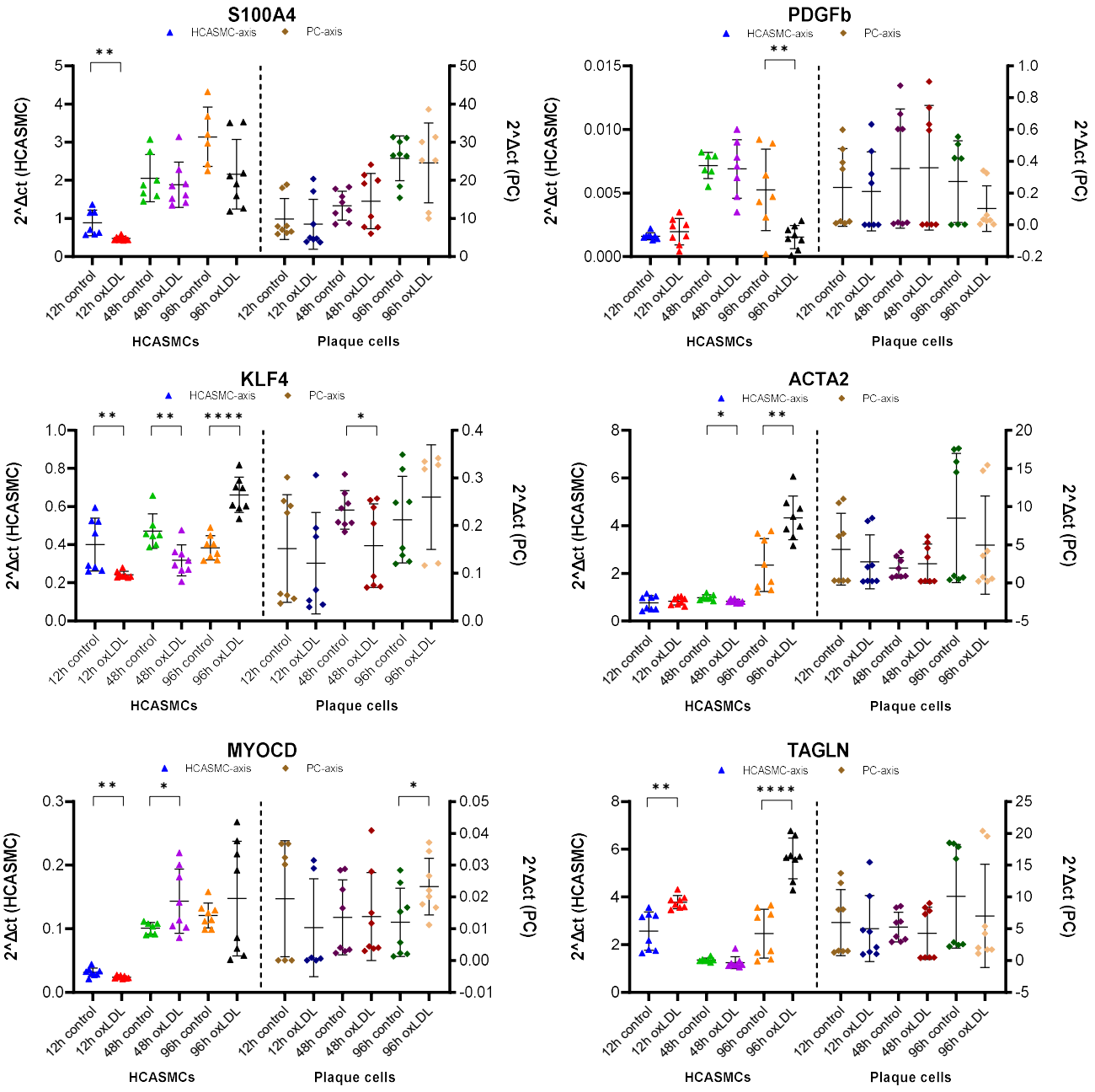

Figure 6: Normalized gene expression of VSMC markers in HCASMCs and plaque cells after oxLDL exposure. Data points (mean ± SD) from 2 different plaque cell lines (N=2) and 2 HCASMC lines (N=2). Comparisons were performed by using unpaired t-test. Ns p>0.05, * p<0.05, ** p<0.01. (HCASMC, human coronary artery smooth muscle cell; PC, plaque cell; oxLDL, oxidised low-density lipoprotein)

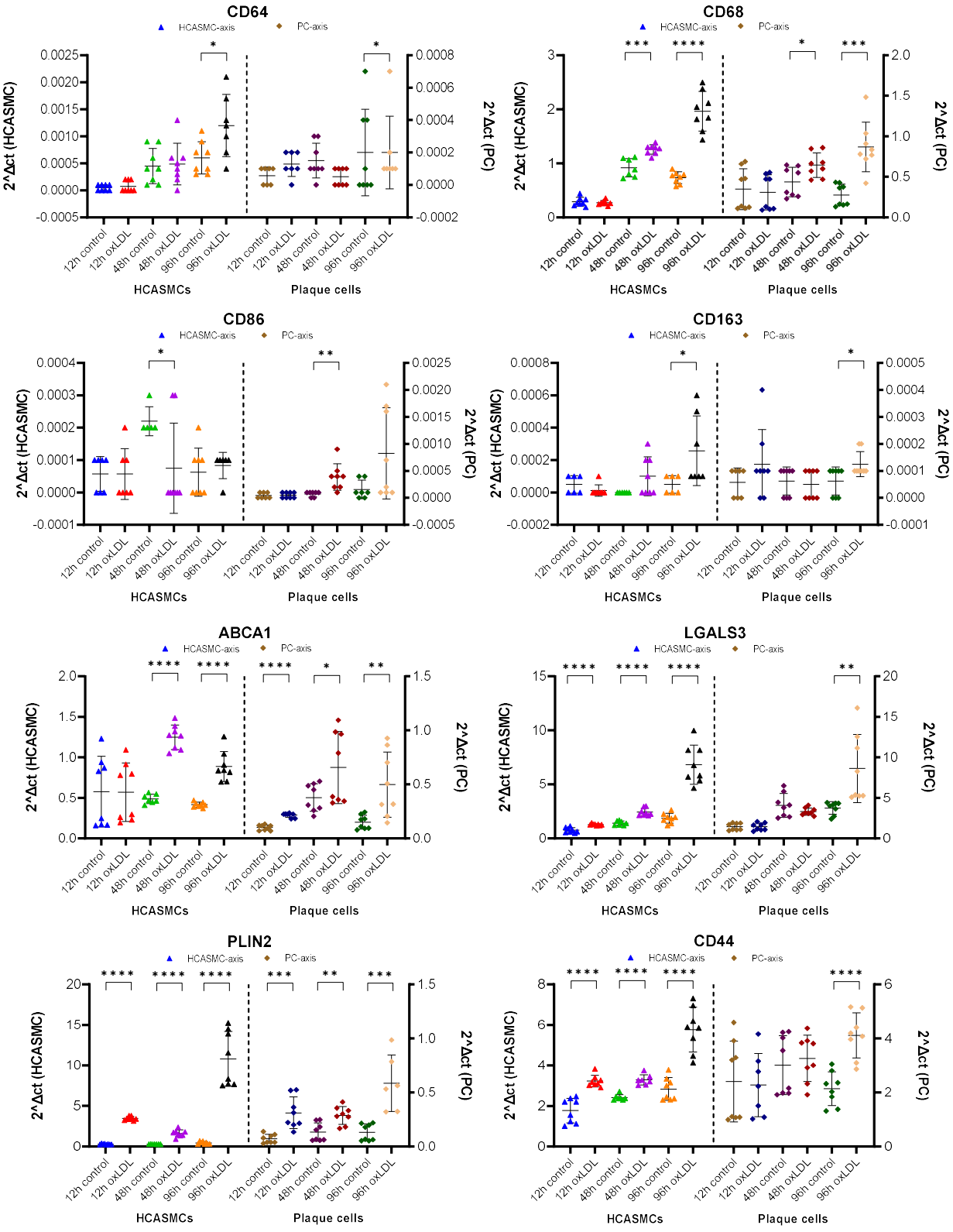

Figure 7: Normalized gene expression of foam cell markers in HCASMCs and plaque cells after oxLDL exposure. Data points (mean ± SD) from 2 different plaque cell lines (N=2) and 2 HCASMC lines (N=2). Comparisons were performed by using unpaired t-test. Ns p>0.05, * p<0.05, ** p<0.01. (HCASMC, human coronary artery smooth muscle cell; PC, plaque cell; oxLDL, oxidised low-density lipoprotein)

3. DISCUSSION

3.1. BIOLOGICAL IMPLICATIONS

The aim of this study was to understand the ability of plaque cells to transdifferentiate toward the foam cell lineage. Foam cells play an important role in the development of the atherosclerotic plaque by forming the lipid core. It is known that macrophages and smooth muscle cells have the ability to take up oxLDL and become foam cells (4,10–17). However, it is not known if plaque cells can also become foam cells via this process.

First, the growing rate and stability of plaque cells were assessed, because plaque cells are primary cells and therefore have a limited proliferative capacity. The plaque cells showed a constant growing rate until passage 8, after which the proliferative capacity decreased. However, HCASMCs grow at a constant growing rate up to passage 11. Next to the proliferation rate, the gene expression profile for canonical SMC markers in plaque cells also did not change significantly over passage 2 to 10. Some passages of CALD1, MYH10, PDGFβ, and KLF4 showed increased fold changes. All in all, plaque cells show an overall stability for the selected SMC markers, together with a stable proliferation rate up to passage 8. However, HCASMCs show more variability in the over time expression of canonical SMC markers, especially two genes, MYH11 and PDGFβ, have been showing a trend over time.

Next, the capability of plaque cells to take up oxLDL was assessed. Both fluorescent images and quantification of the oxLDL uptake showed that plaque cells are capable of taking up oxLDL. Moreover, the oxLDL uptake by plaque cells is dependent on oxLDL concentration and time.

In order to stimulate the plaque cells to transdifferentiate toward the foam cell lineage, multiple conditions were tested. For both plaque cells and HCASMCs the preferred condition is no stimulation, which means only exposure to oxLDL. Stimulation with PDGFβ, S100A4, or the combination of PDGFβ and S100A4 resulted in no extra phenotypic change in the plaque cells. This can be explained by the fact that Sakic *et al.* (20) used oligomeric S100A4 and monomeric S100A4 was used in this study. The researchers described that dimeric or monomeric S100A4 probably would not result in the same phenotypic switch as they found with oligomeric S100A4. Furthermore, PDGFβ alone was not likely to induce a phenotypic switch. This protein is known to stimulate proliferation, but in combination with S100A4 could induce a phenotypic switch. Due to a monomeric S100A4, the stimulation was not successful. However, the plaque cells were still able to show a phenotypic change without stimulation, and only with oxLDL. Secondly, starvation medium and FCS-depletion medium were used to compare the effect of the medium. Both media did not include lipids that could interfere with the oxLDL experiment. Furthermore, in the starvation medium more components were left out to see if the plaque cells would need these to transdifferentiate to a foam-like cell. FCS-depletion medium showed the most phenotypically changed plaque cells and HCASMCs. This is probably because cells plated in starvation medium became too stressed and therefore died. Further comparison of the conditions resulted in the conclusion that the preferred conditions to transdifferentiate plaque cells to foam-like cells are an oxLDL concentration of 50 µg/ml with an incubation time of 96 hours. Overall, the best conditions to transdifferentiate plaque cells toward foam-like cells are no stimulation, in FCS-depletion medium, with 50 µg/ml of oxLDL, and incubated for 96 hours. Another finding was that plaque cells show transdifferentiation after 96 hours, while HCASMCs have a little change in morphology after 24 hours of incubation with oxLDL and stay similar in the following timepoints. Plaque cells however, start to change after 72 hours, with a peak at 96 hours and after this the they started dying. This could indicate that plaque cells and HCASMCs deal differently with oxLDL. Plaque cells originate from an atherosclerotic plaque, which is a stressful environment. The atherosclerotic tissue is dense and lacks essential nutrients and growth factors. This results in a modulation of the VSCMs and VSMC-derived plaque cells. This modulation could explain why HCASMCs change a little after 24 hours and plaque cells transdifferentiate after 72 to 96 hours of exposure to oxLDL. Furthermore, the observed change in morphology of HCASMCs may be explained by the stress environment the HCASMCs have been exposed to during culturing.

Subsequently, the expression of a selection of foam cell and VSMC markers after oxLDL exposure was determined. The selected VSMC markers showed an overall stable expression in plaque cells compared between the oxLDL and control condition. However, the foam cell markers showed significant changes. A significant gene expression change in the expression of CD86 was observed. This gene became upregulated in plaque cells, but not in HCASMCs. It is shown that oxLDL induces an upregulation of CD86, but also that this is a TLR2, TLR4, and CD36 dependent process. This means that activation and upregulation of TLR2, TLR4, and CD36 are essential for the upregulation of CD86 and the formation of foam cells (24). The main difference between plaque cells and HCASMCs is the environment they originate from. Plaque cells are derived from an inflammatory environment in which their inflammatory pathways could have become activated. This includes the activation and upregulation of TRL2, TLR4, and CD36. The different origins of the cells could therefore explain the difference in CD86 gene expression patterns. Another gene that had been highly upregulated after oxLDL exposure was PLIN2. This gene codes for a structural protein in lipid droplets and plays an important role in lipid handling in macrophages by facilitating cholesterol storage and hindering cholesterol efflux (25–27). PLIN2 levels are known to increase after exposure to LDL, because lipids induce PLIN2 expression and the PLIN2 protein is stabilized when it is bound to lipid droplets (28). This makes PLIN2 a marker of lipid accumulation in cells. The upregulation of PLIN2 would inhibit the cholesterol efflux, which stimulates the switch toward a foam cell. Furthermore, Paul *et al.* showed that PLIN2 inactivation in macrophages prevents the formation of foam cells by impairing the build-up of LDL (26). Son *et al.* also found that targeting PLIN2 is well tolerated by macrophages (25), which could mean that PLIN2 is a promising therapeutic target in the treatment of atherosclerosis.

3.2. Limitations and future perspectives

The main limitation of this study is the huge variability between patients. Plaque cells from three different patients were exposed to oxLDL, but they did not respond in the same way. In order to confirm the ability of plaque cells to transdifferentiate to foam cells, the same experiment should be performed on more plaque cell lines. Another limitation is the limited number of markers checked by qPCR. To validate the transcriptional switch found in plaque cells after oxLDL exposure, RNA sequencing would be relevant to perform. This would provide a whole transcriptional picture of this process. One of the most important findings in this study was the clear upregulation of PLIN2 after exposure to oxLDL. PLIN2 could therefore be a specific foam cell marker and might be used as a target to identify and likely prevent the transdifferentiation of plaque cells toward foam cells. However, further studies will be needed to look into this transdifferentiation process. Furthermore, studying male and female plaque cells and how they deal with oxLDL would be interesting. Especially considering the differences between plaque phenotypes and the fact that the unstable plaques are more prevalent in men while the stable ones are more prevalent in females (29).

3.3. Conclusion

The observed phenotypic switching and transcriptional changes of plaque cells confirmed the hypothesis. Plaque cells have the ability to transdifferentiate both in phenotype and gene expression toward a foam cell lineage after exposure to oxLDL. This is important in understanding the role of VSMCs and foam cells in the development of atherosclerosis. Furthermore, it increases the knowledge of plaque progression mechanisms and aids in the search for novel patient-specific therapeutic targets.

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6. METHODS

6.1. CONDITIONED OUTGROW ISOLATION AND CULTURE OF HUMAN PRIMARY PLAQUE CELLS

Human primary plaque cells were isolated from atherosclerotic plaques from patients who just underwent CEA or TEA. Informed consent was obtained from all patients preoperatively. The fresh atherosclerotic tissue from the patient was collected in Hank's Balanced Salt Solution 1X (HBSS, cat. 14025-50, Gibco) on ice within 30 minutes of operative resection.

Under sterile conditions, the direction of the plaque was identified, and the culprit lesion and the next upstream or downstream segment were cut out and processed according to relevant protocols for inclusion in the AtheroExpress biobank (21). These segments were therefore not used in plaque cell isolation. The remaining pieces were cut into 2-3 mm^3 pieces. Subsequently, the pieces were washed 3x with HBSS 1x (Gibco) until the solution ran clear and one or two plaque pieces per well were placed in a 12-well plate, which was pre-coated with 2 µg/cm^2 Fibronectin (cat. F1141, Sigma Aldrich). The pieces were cultured for 14 days, the first 7 days in HAM F12K Complete containing antimicrobial agent for primary cells (Primocin, cat. ant-pm2, InvivoGen) (1:500) and then in complete medium only. The pieces were left at 37°C for 3 days, after which the antibiotic medium was refreshed with freshly made antibiotic medium and incubated for 2 days at 37°C. From day 6 to 14, the complete medium was refreshed every second day. On day 14, the pieces of plaque were removed, and the medium was refreshed every 2 days until at least day 21. On day 21, the cells could be replated and subcultured.

Plaque cells were cultured by refreshing the complete medium one other day and subcultured when the cells reached 70-80% confluency at the density of 5 × 105 cells per T75 dish. Cell counts were determined using the TC20 (Bio-Rad Laboratories, Hercules, CA, USA).

6.2. CELL CULTURE MEDIA

HAM F12K COMPLETE MEDIUM

HAM's F12K (Kaighn's) Nut mix 1x (cat. 21127022, Gibco) supplemented with 10% (v/v) Heat Inactivated fetal calf serum (HI-FCS, Corning), 1% (v/v) Insulin Transferrin Sodium Selenite solution (ITS, cat. 41400045, Fisher Scientific), 1% (v/v) Penicillin-Streptomycin (10,000 U/ml, cat. 15140122, Gibco), 10 mM HEPES (cat. 15630056, Fisher Scientific), 10 mM TES (cat. T1375, Sigma Aldrich), 30µg/ml Endothelial Cell Growth Factors (ECGS, cat. 02-102, Merck/Sigma Aldrich), 2.5µg/ml of Vitamin C solution (L-Ascorbic Acid, cat. A4544, Sigma Aldrich). Vitamin C solution has been added weekly freshly made upon use.

FCS-DEPLETION MEDIUM

It consists of basal medium HAM's F12K (Kaighn's) Nut mix 1x supplemented with 2% (v/v) Insulin Transferrin Sodium Selenite solution, 20 mM HEPES, 20 mM TES, 60 µg/ml Endothelial Cell Growth Factors, 5 µg/ml of Vitamin C solution (added weekly and freshly made upon use).

STARVATION MEDIUM

HAM's F12K (Kaighn's) Nut mix 1x (cat. 21127022, Gibco) supplemented with 1% (v/v) Penicillin-Streptomycin (10,000 U/ml, cat. 15140122, Gibco), 10 mM HEPES (cat. 15630056, Fisher Scientific), and 10 mM TES (cat. T1375, Sigma Aldrich).

6.3. GENERATION OF GROWTH CURVES

Cells were subcultured when 70-80% confluency was reached at the density of 5 × 10^5 cells per 75 cm^2. After this, the cells were subcultured and the passage increased by 1. The cells were used from passage 2 until they showed a significant decrease in proliferation. The number of days between two subculturing procedures was annotated during the cultures. Data were plotted in GraphPad Prism (V9).

6.4. GENE EXPRESSION OF CANONICAL SMC MARKERS TO STUDY STABILITY OVER TIME

During every subculturing step of the plaque cells and HASMCs, samples of 3 x 10^5 plaque cells were collected, lysed and stored at -80°C in 350 µl of lysis buffer (RA1, cat. 750961, Macherey-Nagel). Total RNA was isolated according to the supplier's protocol (Nucleospin RNA, Macherey-Nagel). Transcription of 300 ng of DNA-free RNA into cDNA was performed using the qScript cDNA Synthesis Kit (Quantabio, #95047). qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) with specific primers in a CFX96 Touch Real-Time PCR detection system (Bio-Rad): 5 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, 30 seconds at specific annealing temperature, and 45 seconds at 72°C, followed by melting curve analysis to confirm single product amplification. Messenger RNA (mRNA) expression levels were normalized to human heterochromatin protein 1 binding protein 3 (hHP1BP3) reference gene mRNA expression (ΔCt). Relative differences were calculated (ΔΔCt) and presented as fold induction ($2^{-\Delta\Delta Ct}$). Used primers are shown in Table S1 and S2. Data from 5 different plaque cell lines were used to assess up/down-regulation of canonical SMC markers when compared with commercially available human coronary artery smooth muscle cells (HCASMCs). Data were plotted in GraphPad Prism (V9).

6.5. OXLDL UPTAKE IMAGING AND QUANTIFICATION

Plaque cells and HASMCs have been seeded (2×10^4 / well) into a black 96-well plate and maintained in complete medium for 24 hours (Figure S6A). After 24 hours, the medium was refreshed and replaced with starvation medium. After another 24 hours the medium was refreshed and pH-sensitive conjugated oxidized lipoprotein (oxLDL, L34358, Thermo Fisher Scientific) was added to the cultures. 4 different oxLDL concentrations were used: 2.5, 5, 12.5, and 25 µg/ml. The cells were incubated at 37 °C, 5% CO_2 for either 6, 12, 24, 48, 72, or 96 hours.

After, plaque cells were rinsed 3 times with 1X PBS (Gibco) and fixed for 15 min with 4% paraformaldehyde (PFA, Klinipath). After 3 washing steps with PBS, cells were incubated with DAPI (1:10000) for 3 minutes on a shaker at room temperature in the dark. After, the cells were washed with PBS 3 times and imaged using a fluorescence microscope with transmitted light filter for cell morphology images, DAPI filter for nuclei visualization, and RFP filter for lipid visualization (EVOS FL, 336595). The oxLDL taken up by the cells was quantified with ImageJ using a macro run protocol (Figure S7)(30). The control conditions, cells without any exposure to oxLDL, were used as threshold for the blue (DAPI) and red (lipids) imaging parameters. The taken up oxLDL was divided by the number of nuclei to account for the number of cells on the image. Data from 2 different plaque cell lines (each 2 wells and each 3 images) were used to assess the oxLDL uptake per cell. Data were plotted in GraphPad Prism (V9).

6.6. Establishing conditions to transdifferentiate plaque cells to foam cell lineage

Plaque cells and HASMCs have been seeded (1×10^4 / well) into a 96-well plate and maintained in complete medium for 24 hours (Figure S6B). Following, pre-stimulation started with the addition of either PDGFβ (30 ng/ml), S100A4 (2,5 µg/ml; cat: 4137-S4, R&D Systems), or the combination of PDGFβ and S100A4 in complete medium and were left for 4 days. After 4 days of incubation, the medium was replaced with either FCS-depletion or starvation medium and was left for 24 hours and 100% confluency was accomplished. After 24 hours the medium was refreshed with FCS-depletion or starvation medium and oxidized lipoprotein (oxLDL, L34357, Thermo Fisher Scientific) was added to the cultures. 3 different oxLDL concentrations were used: 12.5, 25, and 50 µg/ml. The cells were incubated at 37 °C, 5% CO_2 for either 24, 48, 72, 96, or 120 hours. After, the cells were imaged using a fluorescence microscope with transmitted light filter for cell morphology images (EVOS FL, 336595).

6.7. Gene expression of VSMC and foam cell markers to study change in expression after oxLDL incubation

Samples of plaque cells and HCASMCs were collected, lysed and stored at -80°C in 350 µl of lysis buffer (RA1, cat. 750961, Macherey-Nagel) after 12, 48, and 96 hours of oxLDL exposure (no pre-stimulation, in FCS-depletion medium) (Figure S6C). For every timepoint a control condition without oxLDL was included. Total RNA was isolated according to the supplier's protocol (Nucleospin RNA, Macherey-Nagel). Transcription of 300 ng of DNA-free RNA into cDNA was performed using the qScript cDNA Synthesis Kit (Quantabio, #95047). qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad) with specific primers in a CFX96 Touch Real-Time PCR detection system (Bio-Rad): 5 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at specific annealing temperature, and 45 s at 72°C, followed by melting curve analysis to confirm single product amplification. Messenger RNA (mRNA) expression levels were normalized to human heterochromatin protein 1 binding protein 3 (hHP1BP3) reference gene mRNA expression (ΔCt). Relative differences were calculated (ΔΔCt) and presented. Primers used are shown in Table S1 and S2. Data from 2 different plaque cell lines and HCASMCs were used to assess up/down-regulation of foam cell and VSMC gene markers. Data were plotted in GraphPad Prism (V9) in which the significant difference was calculated between the oxLDL and control condition.

6.8. Statistical analysis

Graphical representation and statistical analysis of qPCR and oxLDL uptake data were obtained using GraphPad Prism V9. Unpaired t-test was used to compare the experimental groups with the control groups. Barplot data are presented as mean ± standard deviation (SD). A p-value ≤ 0.05 was deemed statistically significant with * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.

7. Supplementary Materials

Table S1: Genes used in gene expression experiments.

Gene Name	Used in experiment	Specificity
ABCA1	oxLDL	Foam cell
ACTA2	Stability and oxLDL	SMC
CALD1	Stability	SMC
CD44	oxLDL	Foam cell
CD64	oxLDL	Foam cell
CD68	oxLDL	Foam cell
CD86	oxLDL	Foam cell
CD163	oxLDL	Foam cell
CNN1	Stability	SMC
KLF4	Stability and oxLDL	SMC
LGALS3	oxLDL	Foam cell
MMP2	Stability	SMC
MYH10	Stability	SMC
MYH11	Stability	SMC
MYOCD	Stability and oxLDL	SMC
PDGFβ	Stability and oxLDL	SMC
PLIN2	oxLDL	Foam cell
S100A4	oxLDL	SMC
TAGLN	Stability and oxLDL	SMC
TPM4	Stability	SMC

Table S2: Primers used in gene expression experiments.

Gene Name	Forward Primer	Reverse Primer	Ampli-cone (bp)	Gene-ID NCBI	Annealing Temp (°C)
hHP1BP3	CCCACGTCCCAAGATGGAT	CTGATGCACCACTCTTCTGGAA	71	50809	60.5 - 62.6
ABCA1	ACATCCTGAAGCCAATCCTGA	CTCCTGTCGCATGTCACTCC	152	19	60.5
ACTA2	CTATGAGGGCTATGCCTTGCC	GCTCAGCAGTAGTAACGAAGGA	122	59	60.5
CALD1	TCGACCCATCAAGCAATA	CCGGCTTTGTAGGTTTTGCG	100	800	62.6
CD44	CTGCCGCTTTGCAGGTGTA	CATTGTGGGCAAGGTGCTATT	109	960	60.5
CD64	GCATGGGAAAGCATCGCTAC	GCAAGAGCAACTTTGTTTCACA	147	2209	60.5
CD68	CTTCTCTCATTCCCCTATGGACA	GAAGGACACATTGTACTCCACC	105	968	60.5
CD86	CTGCTCATCTATACACGGTTACC	GGAAACGTCGTACAGTTCTGTG	133	942	60.5
CD163	TTTGTCAACTTGAGTCCCTTCAC	TCCCGCTACACTTGTTTTCAC	127	9332	60.5
CNN1	CTGTCAGCCGAGGTTAAGAAC	GAGGCCGTCCATGAAGTTGTT	123	1264	62.6
KLF4	CCCACATGAAGCGACTTCCC	CAGGTCCAGGAGATCGTTGAA	170	9314	60.5 - 62.6
LGALS3	GTGAAGCCCAATGCAAACAGA	AGCGTGGGTTAAAGTGGAAGG	76	3958	60.5
MMP2	GATACCCCTTTGACGGTAAGGA	CCTTCTCCCAAGGTCCATAGC	112	4313	60.5
MYH10	GCAGGAGAACACCTAAAGTCTG	TGTCCCGGAATAGGAATATAGCC	89	4628	62.6
MYH11	CGCCAAGAGACTCGTCTGG	TCTTTCCCAACCGTGACCTTC	129	4629	62.6
MYOCD	CCACCTATGGACTCAGCCTAC	CTCAGTGGCGTTGAAGAAGAG	188	93649	60.5 - 62.6
PDGFβ	CTCGATCCGCTCCTTTGATGA	CGTTGGTGCGGTCTATGAG	239	5155	60.5
PLIN2	TTGCAGTTGCCAATACCTATGC	CCAAGTCACAGTAGTCGTCACA	148	123	60.5
S100A4	GATGAGCAACTTGGACAGCAA	CTGGGCTGCTTATCTGGGAAG	123	6275	60.5
TAGLN	AGTGCAGTCCAAAATCGAGAAG	CTTGCTCAGAATCACGCCAT	154	6876	60.5
TPM4	AATTTGCAGAGAGAACGGTTGC	CAGTGTCTGATGTAAGCCCAC	104	7171	60.5

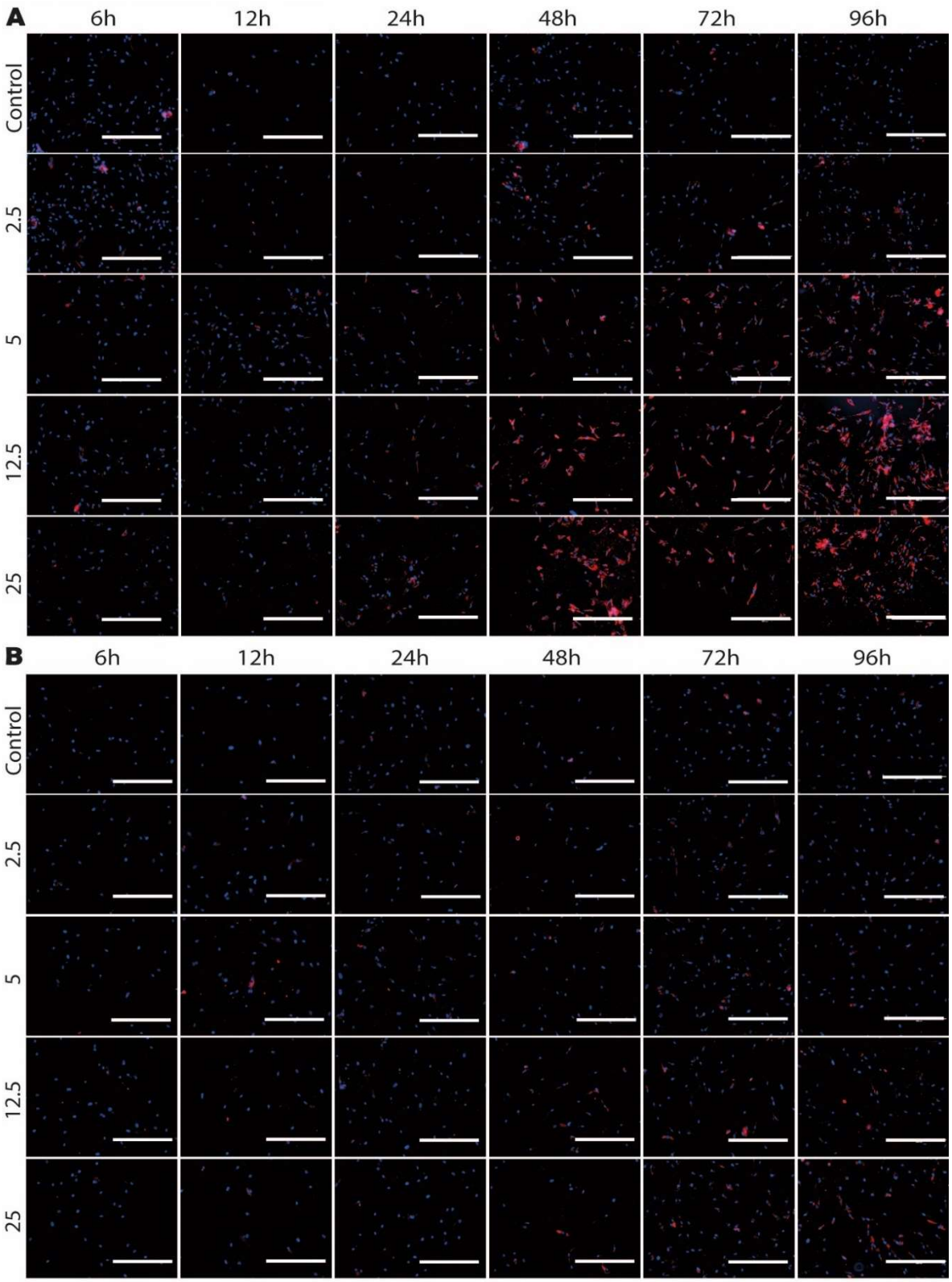

Figure S1: Fluorescent images show oxLDL uptake of plaque cells is dependent on oxLDL concentration and time. Control is defined as no exposure to oxLDL. Vertical numbers represent the oxLDL concentration in µg/ml and horizontal numbers represent the time of incubation with oxLDL. Blue dots stain the nuclei and red dots originate from oxLDL taken up by the cells. Bar = 400 µm. **(A)** Plaque cells from patient AE4674. **(B)** Plaque cells from patient AE4657.

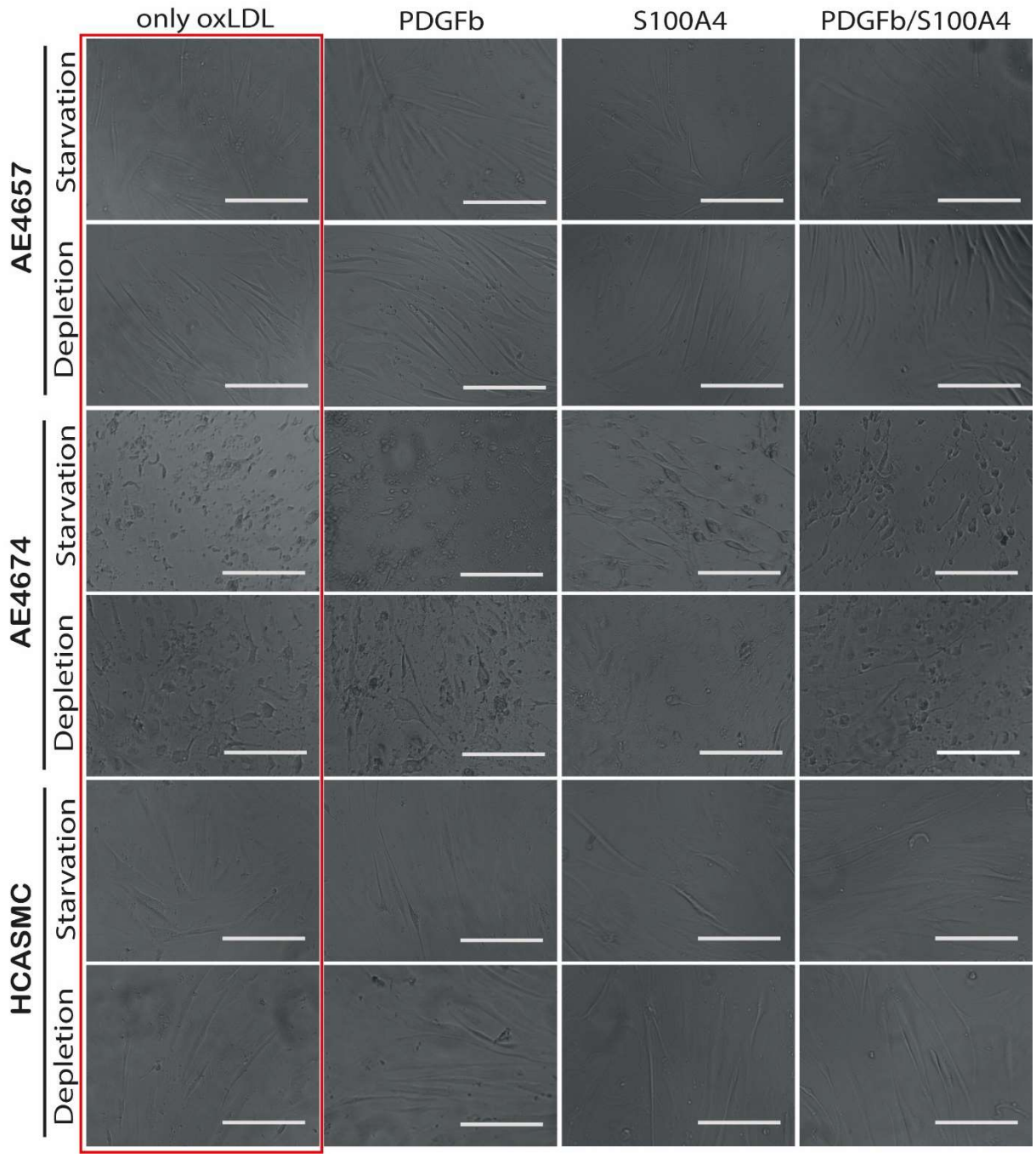

Figure S2: Transmitted light images show comparison of pre-stimulation methods to switch plaque cells toward foam-like cells. Red box shows chosen stimuli (no stimuli, only oxLDL). Cells were incubated with 50 µg/ml oxLDL for 96 hours. Bar = 200 µm. Cells used in this experiment include plaque cells from patient AE4657 and AE4674, and HCASMCs. (oxLDL, oxidated low-density lipoprotein; HCASMC, human coronary artery smooth muscle cell)

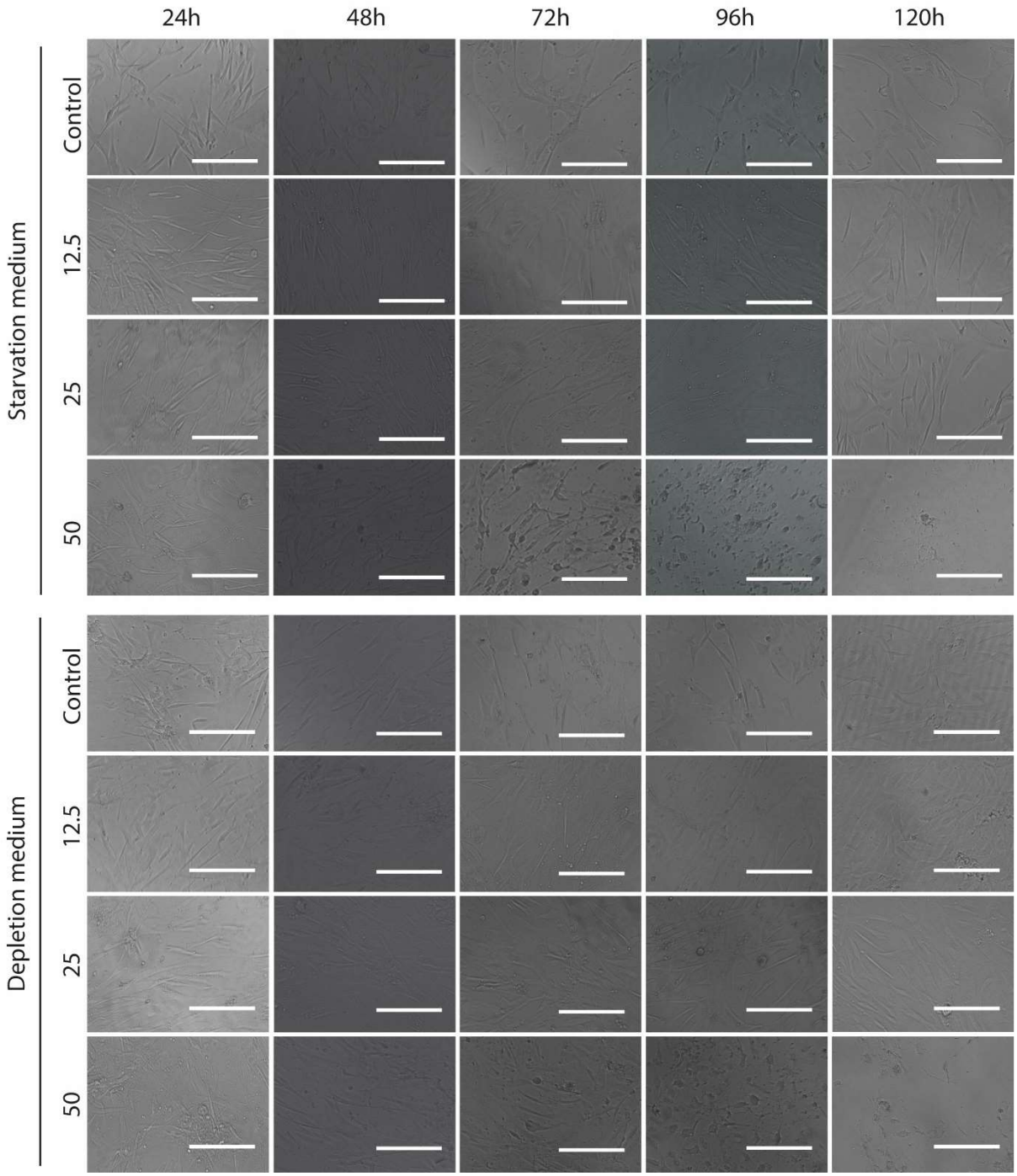

Figure S3: Transmitted light images of plaque cells from patient AE4674. Different oxLDL concentrations (control, 12.5, 25, 50 µg/ml) and different timepoints of incubation (24, 48, 72, 96, 120 hours) in both starvation and FCS-depletion medium are compared to switch plaque cells toward foam-like cells. No pre-stimulation was used. Bar = 200 µm. (oxLDL, oxidated low-density lipoprotein)

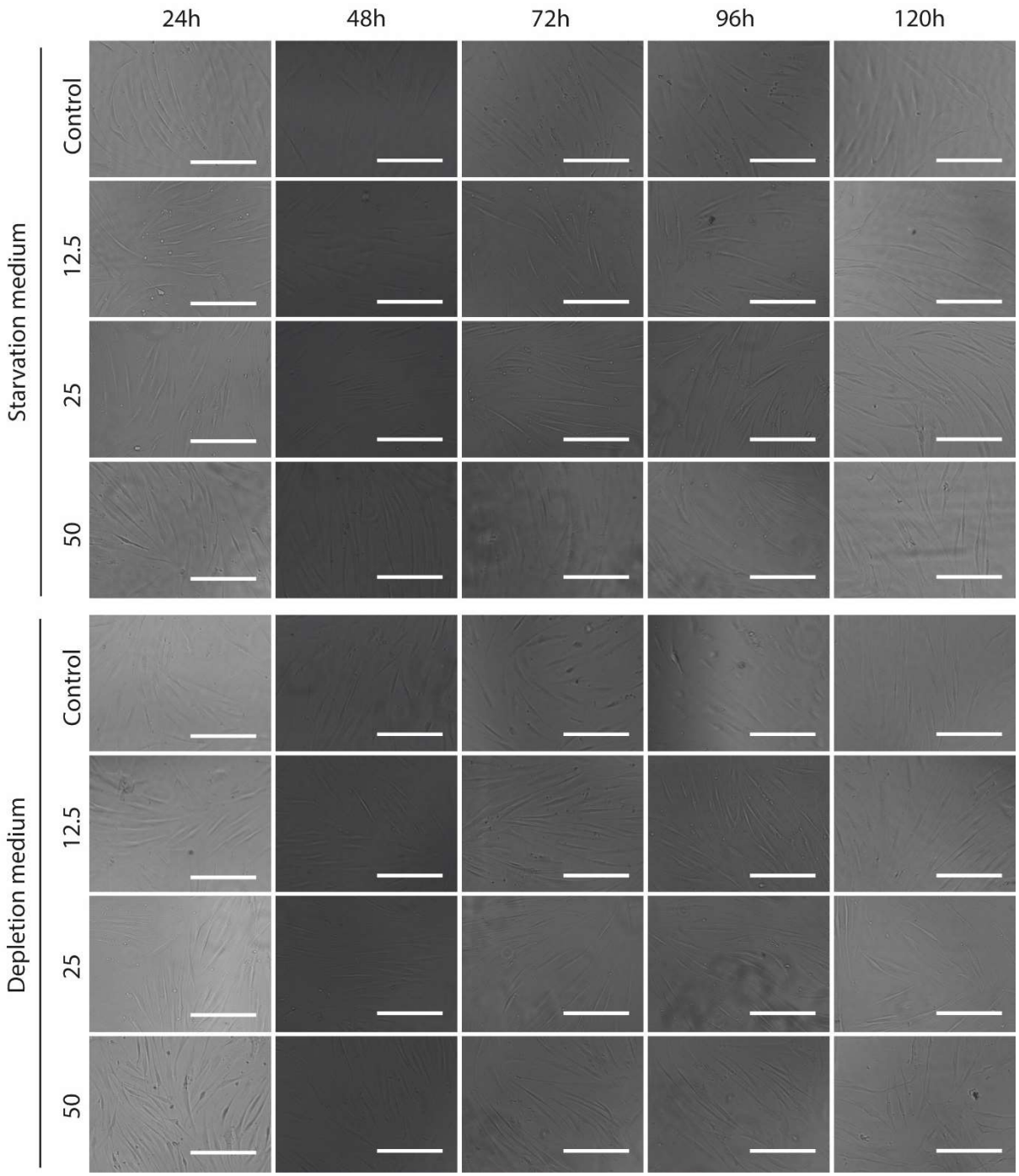

Figure S4: Transmitted light images of plaque cells from patient AE4657. Different oxLDL concentrations (control, 12.5, 25, 50 µg/ml) and different timepoints of incubation (24, 48, 72, 96, 120 hours) in both starvation and FCS-depletion medium are compared to switch plaque cells toward foam-like cells. Bar = 200 µm. (oxLDL, oxidated low-density lipoprotein)

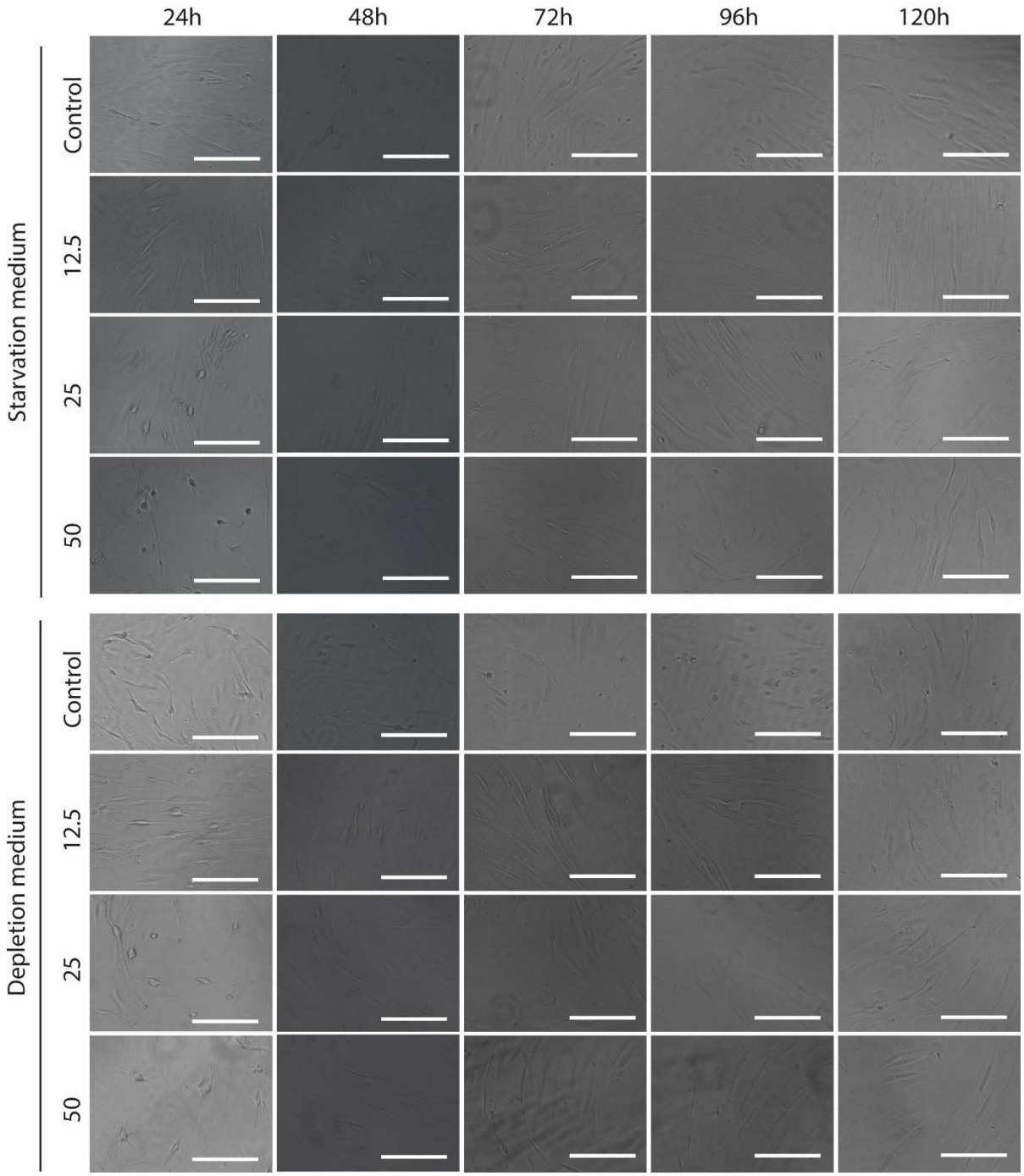

Figure S5: Transmitted light images of HCASMCs. Different oxLDL concentrations (control, 12.5, 25, 50 µg/ml) and different timepoints of incubation (24, 48, 72, 96, 120 hours) in both starvation and FCS-depletion medium are compared to switch plaque cells toward foam-like cells. Bar = 200 µm. (oxLDL, oxidated low-density lipoprotein; HCASMC, human coronary artery smooth muscle cell)

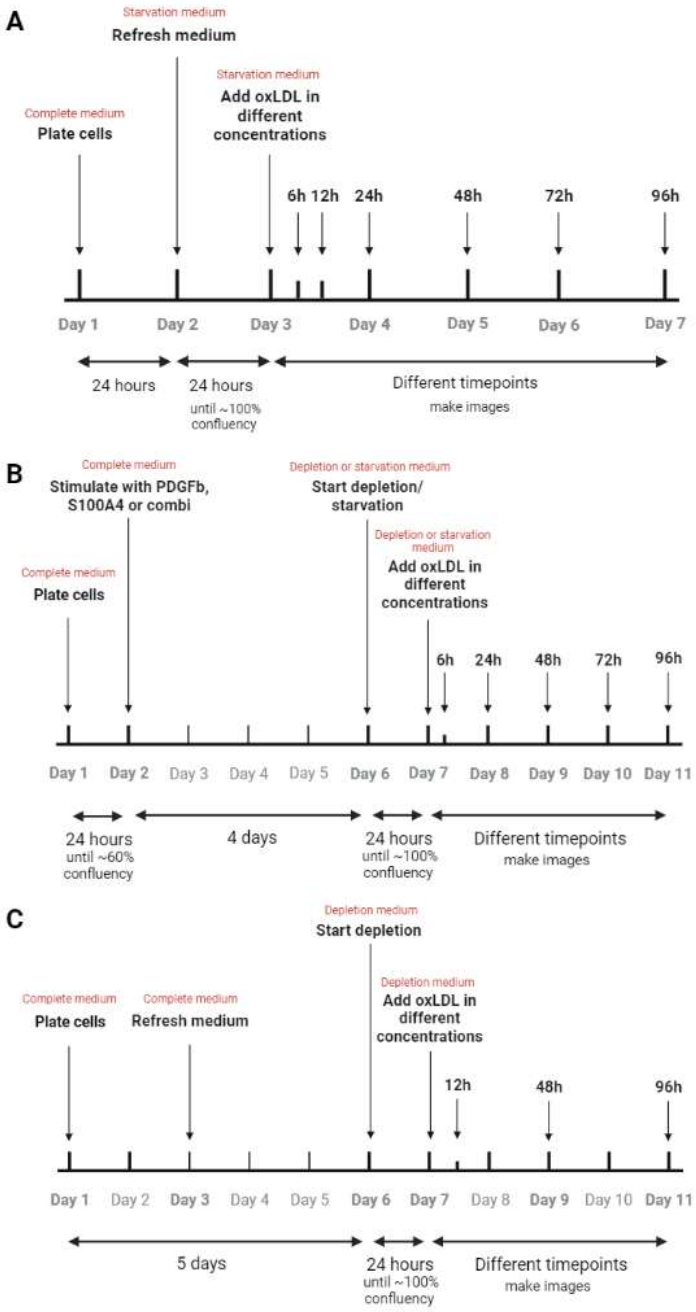

Figure S6: Protocols for oxLDL incubation. (A) Protocol for oxLDL uptake imaging and quantification. **(B)** Protocol to establishing optimal conditions to transdifferentiate plaque cells to foam cell lineage **(C)** Protocol to study change in gene expression after oxLDL incubation. (oxLDL, oxidised low-density lipoprotein)

```
// Set directory and content
extension = ".tif";
dir1 = getDirectory("D:\Staining images\Images script");
setBatchMode(true);
n = 0;
processFolder(dir1);

function processFolder(dir1) {
	list = getFileList(dir1);
	for (i=0; i<list.length; i++) {
		if (endsWith(list[i], "/"))
			processFolder(dir1+list[i]);
    else if (endsWith(list[i], extension))
      processImage(dir1, list[i]);
	}
}

function processImage(dir1, name) {
	open(dir1+name);
  print(n++, name);

title = getTitle();

//setTool("line");
makeLine(767, 846, 1228, 847);
run("Set Scale...", "distance=461.0011 known=200 unit=um");

// Split channels to get green, blue, and red channels separately
run("Split Channels");

// Close the green channel as it's not of interest to us
selectWindow(title + " (green)");
close();

//open file
selectWindow(title + " (blue)");
setAutoThreshold("Default");

//set threshold to analyse blue nuclei. The first number (now 77) can be adjusted so that you see separate cells
setThreshold(77, 255);

// count the number of particles/nuclei/cells
run("Analyze Particles...", "size=100-Infinity circularity 0.00-1.00 summarize add");

//set threshold to analyse red uptake. The first number (now 160) can be adjusted so that you see an amount of red that is
in agreement with the original image
selectWindow(title + " (red)");
setAutoThreshold("Default");
setThreshold(160, 255, "raw");

// return the amount of red, which is the taken up oxLDL
run("Measure");
}
```

Figure S7: Macro run protocol for ImageJ.