APPLICATION NOTE

Phenotypic analysis of hypertrophy in human iPSC-derived cardiomyocytes.

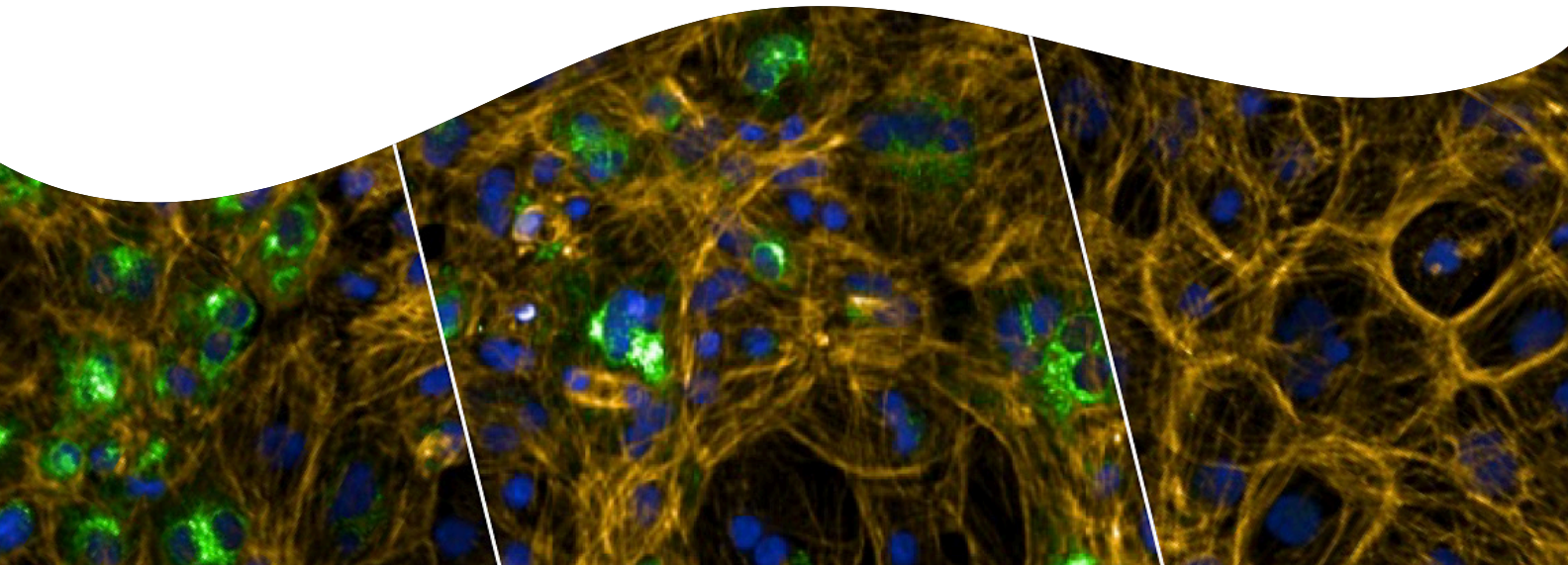
Key points:

- Differentiate early and late effects in endothelin-1-induced hypertrophic cardiomyocytes
- Leverage robust and fast analysis approaches without single cell segmentation
- Analyze phenotypic changes of cardiac hypertrophy with SER and Haralick texture features

Introduction

Cardiac hypertrophy is characterized by an abnormal enlargement of the heart muscle. Common causes include hypertension, diabetes, hypertrophic cardiomyopathy or ischemic diseases. If left untreated it carries the risk of heart failure and can lead to sudden cardiac death.[1,2,3] On a cellular level, hypertrophy is characterized by re-expression of genes normally expressed during fetal development, reorganization of cytoskeletal proteins and an increase in size of cardiomyocytes. Successful drug discovery campaigns for hypertrophy depend on physiologically relevant cell culture systems, ideally based on cardiomyocytes of human origin. However, human primary cardiomyocytes are not readily available, are difficult to maintain in culture and cannot be propagated as they do not proliferate. A new and more accessible source of cells for cardiac disease modeling is human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes.

In this study, Cor.4U® hiPSC-derived cardiomyocytes from Ncardia were used to set up a phenotypic assay on the Opera Phenix® Plus high-content screening system to study time-dependent effects of ET-1 (endothelin-1)-induced hypertrophy. A high proBNP expression level was identified as an early marker for hypertrophy, while changes in the cytoskeletal organization became visible at a later time point, by quantifying advanced SER and Haralick texture features using Harmony® high-content imaging and analysis software.



ProBNP upregulation during endothelin-1-induced hypertrophy

One of the hallmarks of hypertrophy is the re-expression of fetal genes. One of these genes is brain natriuretic peptide or B-type natriuretic peptide (BNP),[4] a secreted protein. To retain sufficient protein inside the cells for staining, the cells are treated with Brefeldin A to prevent secretion of the pro form of BNP, proBNP, which is cleaved after secretion to form the active BNP. ProBNP levels are also used in diagnostic tests to rule out heart failure.

Cor.4U® iPSC-derived human cardiomyocytes (Ncardia) were plated into a gelatin coated 96-well plate in Cor.4U® Complete Culture Medium (Ncardia) at a density of 30,000 cells per well. On day two, medium was changed to Supplemented Williams E (SWE) medium, a serum free and glucose-reduced medium. On day seven (after five days in SWE medium), cells were stimulated for 18 h with a six point dilution series of endothelin-1 (ET-1), ranging from 3E-12 to 3E-7 M in triplicate. Before fixation, cells were treated for three hours with Brefeldin A to accumulate proBNP inside the cells. After fixation, cells were stained with Hoechst 33342 (Molecular Probes # H3570) and an anti proBNP antibody (Abcam # ab13115), which was visualized with an Alexa 488 conjugated secondary antibody (Invitrogen # A11001). Images were acquired on the Opera Phenix Plus high-content screening system in confocal mode using a 20x water immersion objective.

To analyze the number of proBNP-positive cells, the nuclei were segmented and a ring region around this initial nuclei region was defined. The proBNP staining intensity inside this ring region was calculated and proBNP positive and negative cells were selected based on an intensity threshold. Finally, the percentage of proBNP positive cells was calculated. As shown in Figure 1A, there is a dose dependent increase in the number of proBNP positive cells with an EC_{50} value of 3.1E-10 M ET-1 (Figure 1B). Additionally, the nuclear area was calculated and a significant ET-1-dependent increase in nuclear size of about 15 % was measured which is also described in current literature as a hypertrophy related phenotypic change.[3,5,6]

Texture based analysis of ET-1-induced hypertrophy

Besides the re-expression of fetal genes, cardiac hypertrophy is characterized by changes in the cytoskeletal network. To analyze changes in the actin cytoskeleton, Cor.4U® cardiomyocytes were treated for 18 h and 48 h with a nine-point dilution series of ET-1 ranging from 3E-14 to 3E-6 M. Untreated cells were included as negative control. Cells were stained with rhodamine conjugated phalloidin (Figure 2). Image stacks of five planes at a distance of 2 µm were acquired on the Opera Phenix Plus system using a 20x water immersion objective in confocal mode. Image analysis was done on a maximum intensity projection of the planes. For the texture based approach used here, there is no need to segment individual cells, making this type of analysis less dependent on cell density. To analyze the actin cytoskeleton, the whole of the area covered with cells was detected. Within this area, SER and Haralick texture properties were analyzed. SER texture (Spots, Edges and Ridges) quantifies the occurrence of eight characteristic intensity patterns such as spots, edges and ridges within the image. Haralick texture analysis is based on a co-occurrence matrix and allows, for example, the quantification of homogeneity within an image.[7]

As shown in Figure 2, ET-1 induces a more homogenous distribution of filamentous actin. With SER and Haralick texture properties, clear differences can be detected (Figure 3). The high Z' prime values after both 18 h and 48 h of ET-1 treatment for either the SER Edge (0.67 and 0.61) or Haralick Homogeneity properties (0.65 and 0.76) show that they are robust readout parameters. The dose response curves shown in Figure 3 indicate that changes in the actin network occur already at 18 h and become more pronounced after 48 h. Texture based EC_{50} values after 48 h are in the same concentration range as proBNP intensity-based EC_{50} values after 18 h.

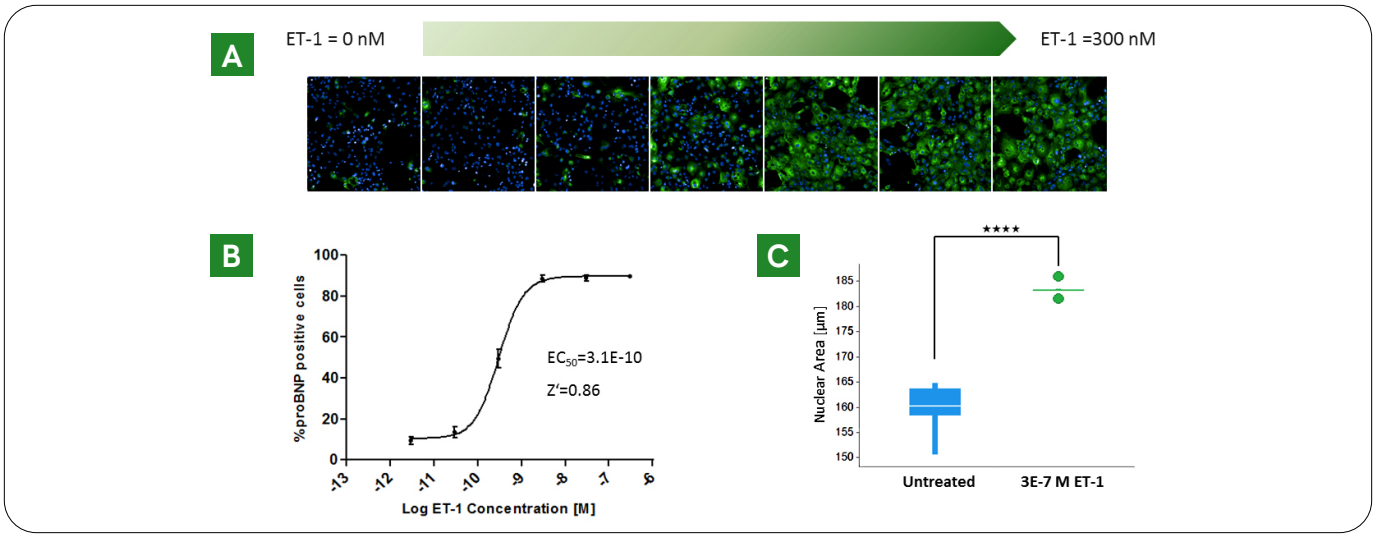


Figure 1. ET-1 induces proBNP expression and increases the nucleus size in iPSC-derived human cardiomyocytes. (A) Example images of cells treated with increasing concentrations of ET-1 for 18 h. Cells were treated with Brefeldin A for three hours prior to fixation and stained for proBNP (green). The proBNP staining intensity increases with increasing ET-1 concentration. (B) Dose response curve for proBNP expression. The percentage of proBNP-positive cells was plotted against the log of ET-1 concentration in [M]. The calculated EC_{50} value is 3.1E - 10M. (C) ET-1 treatment leads to a significant increase in nuclear area of about 15 % ($p < 0.0001$, n = six wells).

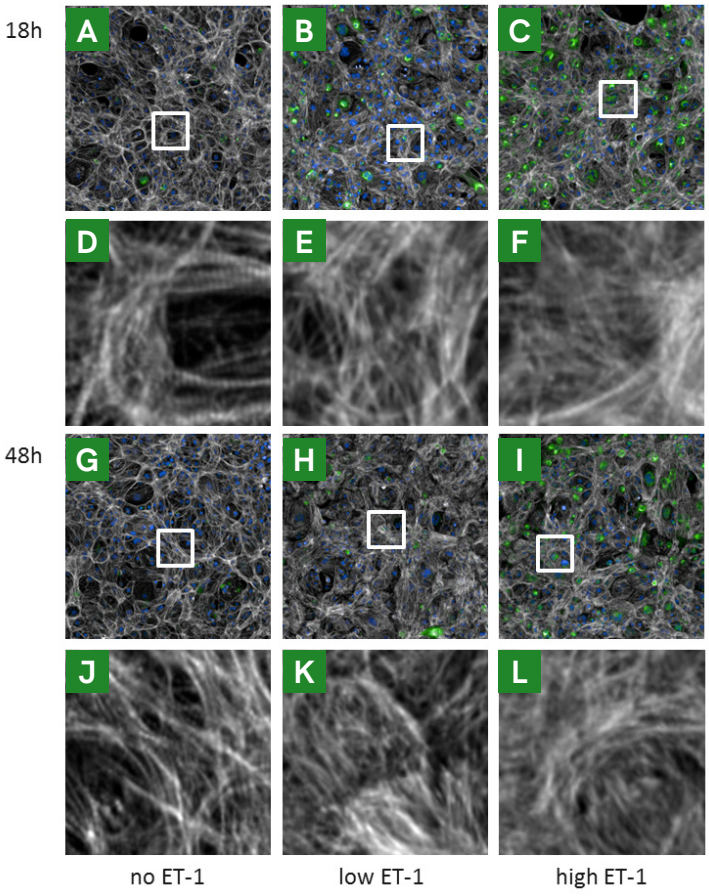


Figure 2. ET-1 induces changes in the filamentous actin network. Cor.4U® hiPSC-derived cardiomyocytes were treated with ET-1 for either 18 h (A-F) or 48 h (G-L) to induce a hypertrophic phenotype and imaged on the Opera Phenix Plus system using a 20x W objective. Shown are example images from untreated, low ET-1 (3E-9 M ET-1) and high ET-1 (3E-7 M ET-1) treated cells. A-C and G-I show merge images of actin (grey), proBNP (green) and nuclei (blue). D-F and J-L are zoomed in images on the indicated regions show-ing filamentous actin only. The filamentous actin network becomes more homogenous after ET-1 treatment.

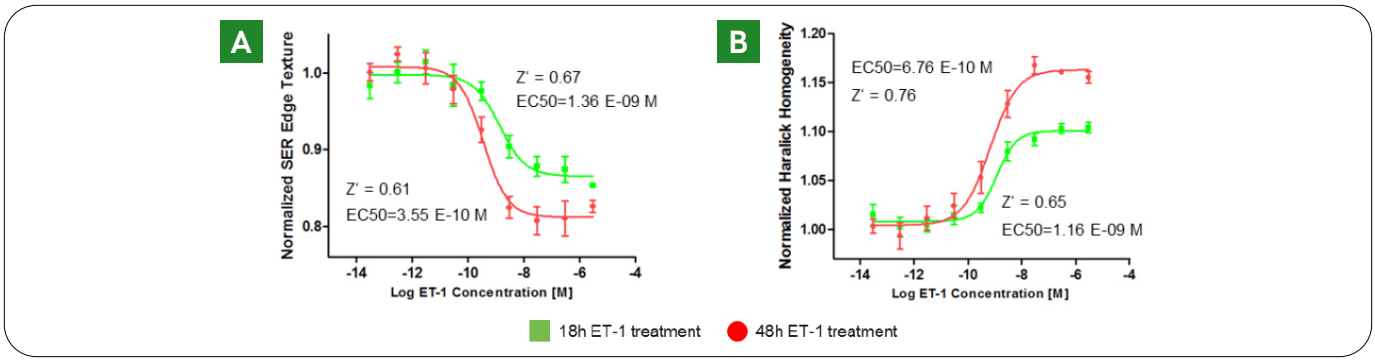


Figure 3. SER and Haralick texture properties allow robust detection of ET-1-induced changes in filamentous actin. Cor.4U® hiPSC-derived cardiomyocytes were treated with ET-1 for either 18 h (green curves) or 48 h (red curves) to induce a hypertrophic phenotype. SER edge (A) and Haralick homogeneity (B) properties were calculated and EC_{50} curves plotted for the two texture properties. 48 h treatment leads to more pronounced changes in the texture properties and to lower EC_{50} values. Both texture properties have high Z′ prime values after either 18 h or 48 h ET-1 treatment.

Conclusion

Cardiac hypertrophy is a major health risk and approaches to discover drugs that would alleviate the hypertrophic phenotype are needed. We have shown here how ET-1-induced hypertrophy in hiPSC-derived cardiomyocytes can easily be analyzed using the Opera Phenix Plus system and Harmony image analysis software.

The assay established here is based on three phenotypic markers: the re-expression of BNP, changes in nuclear size, and changes in the filamentous actin network. The two endpoints tested revealed pro-BNP upregulation and increased nucleus size to be early markers (18 h), whereas changes in the actin network can be detected after 18 h but are more pronounced at a later timepoint (48 h). As expected, the highest Z′ value (0.86) was achieved with proBNP as the readout parameter, but changes in the actin network also resulted in high Z′ values of up to 0.76, suitable for screening.

Furthermore, the increase in nuclear size was of high significance ($p < 0.0001$). It is noteworthy that texture-based analyses do not require single cell segmentation making it a robust, fast and easy approach. Taken together, the Opera Phenix Plus system and Harmony image analysis software allow the phenotypic analysis of complex disease models for cardiac hypertrophy using hiPSC-derived cardiomyocytes.

References

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