Functional Characterization of Three-Dimensional (3D) Human Liver Tissues Generated by an Automated Bioprinting Platform

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Purpose: To create a more physiologically relevant, multicellular liver model to enable investigation of drug-induced liver injury and xenobiotic metabolism.

Abstract: The lack of biological complexity and limited life span of conventional human hepatocyte culture models are major limitations in toxicological research. Recent advances in three-dimensional (3D) monoculture and co-culture systems show promise, but may suffer from the lack of reproducibility and spatial control. In this study, we generated 3D human liver tissue mimetics composed of parenchymal and non-parenchymal cell populations using a proprietary automated 3D fabrication platform. The resulting liver tissues were well organized, containing junctional protein E-Cadherin between hepatocytes, CD31⁺ endothelial cell networks and desmin⁺ quiescent stellates. The hepatocytes also demonstrated the ability to store both lipids and glycogen. The tissues actively secreted albumin, cholesterol, fibrinogen, and transferrin into the medium for six weeks. The 3D liver tissues expressed key Phase I enzymes CYP3A4, 2D6, 2B6, 1A2, and 2C9 over 28 days. Basal activity of CYP3A4, as evidenced by the conversion of midazolam to 4-hydroxymidazolam, was detected throughout the 28 day culture period. In addition, exposure to rifampicin led to an increase in both CYP3A4 mRNA and activity, with 4-fold induction still evident after 28 days in culture. The 3D liver tissues exhibited a clinically relevant injury response, evidenced by decreased overall viability, to published hepatotoxic agents such as Diclofenac and Troglitazone. These results demonstrate the potential utility of human 3D bioprinted liver tissues in drug discovery and development.

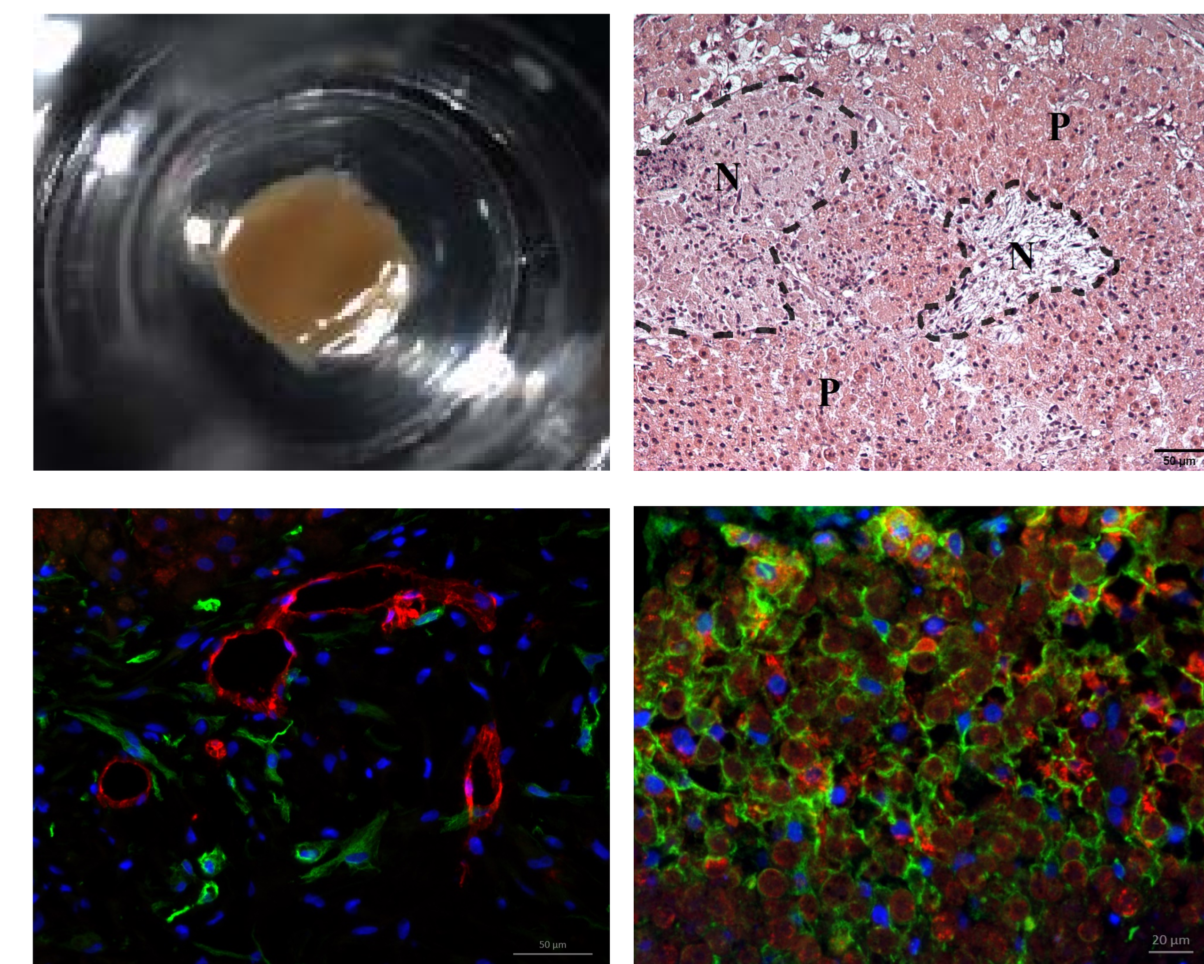

Figure 1: (Top, Left) Representative image of single *exVive3D™* human liver tissue, measuring 2.5 x 2.5mm, with a 0.5mm thickness. Representative images of H&E section (Top, Right) showing distinct zones of non-parenchymal (N) and parenchymal (P) cells, CD31⁺ microvascular structures in close association with Desmin⁺ stellates (Bottom, Left), and E-Cadherin⁺ tight junctions between Albumin⁺ hepatocytes (Bottom, Right).

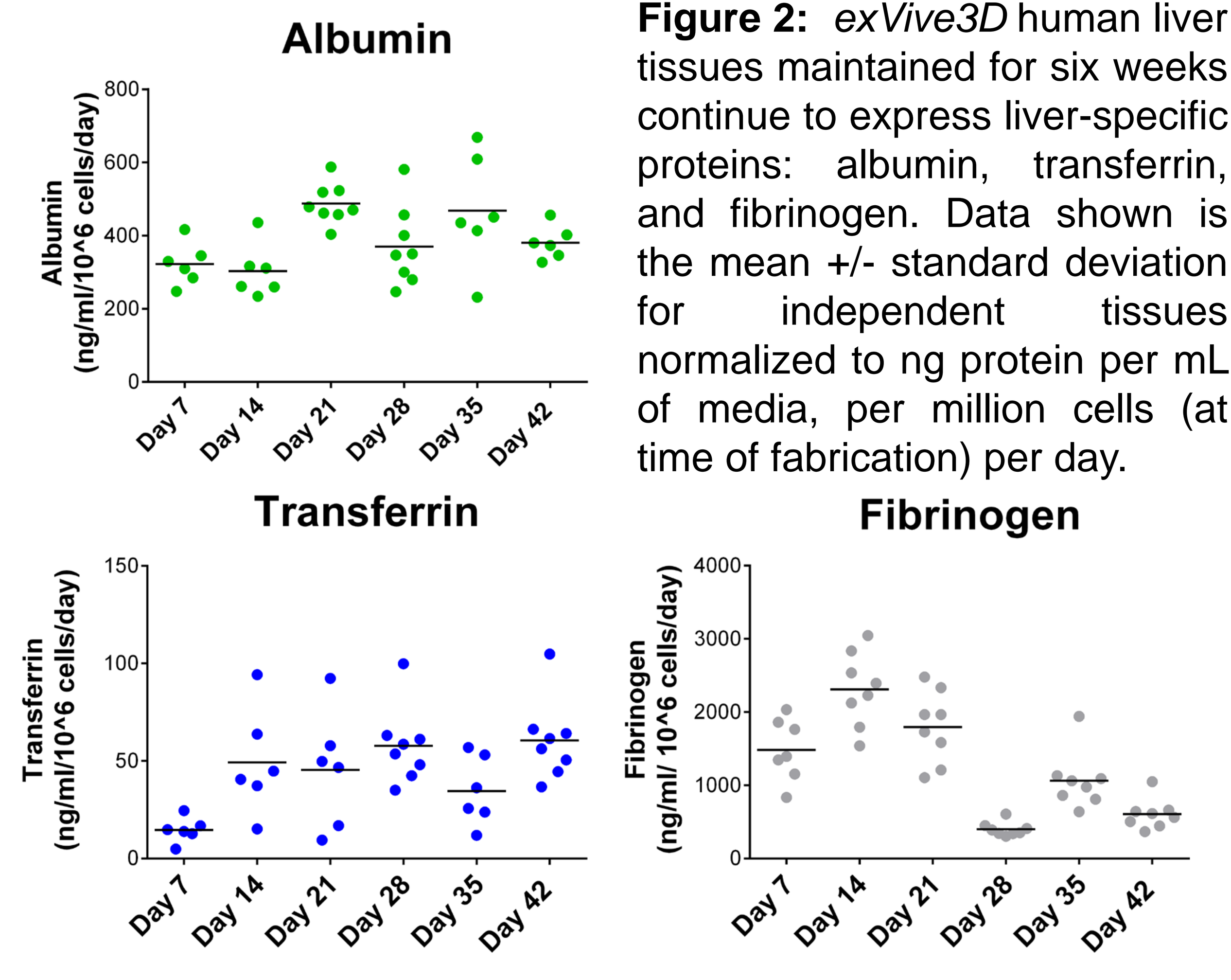

Figure 2: *exVive3D* human liver tissues maintained for six weeks continue to express liver-specific proteins: albumin, transferrin, and fibrinogen. Data shown is the mean +/- standard deviation for independent tissues normalized to ng protein per mL of media, per million cells (at time of fabrication) per day.

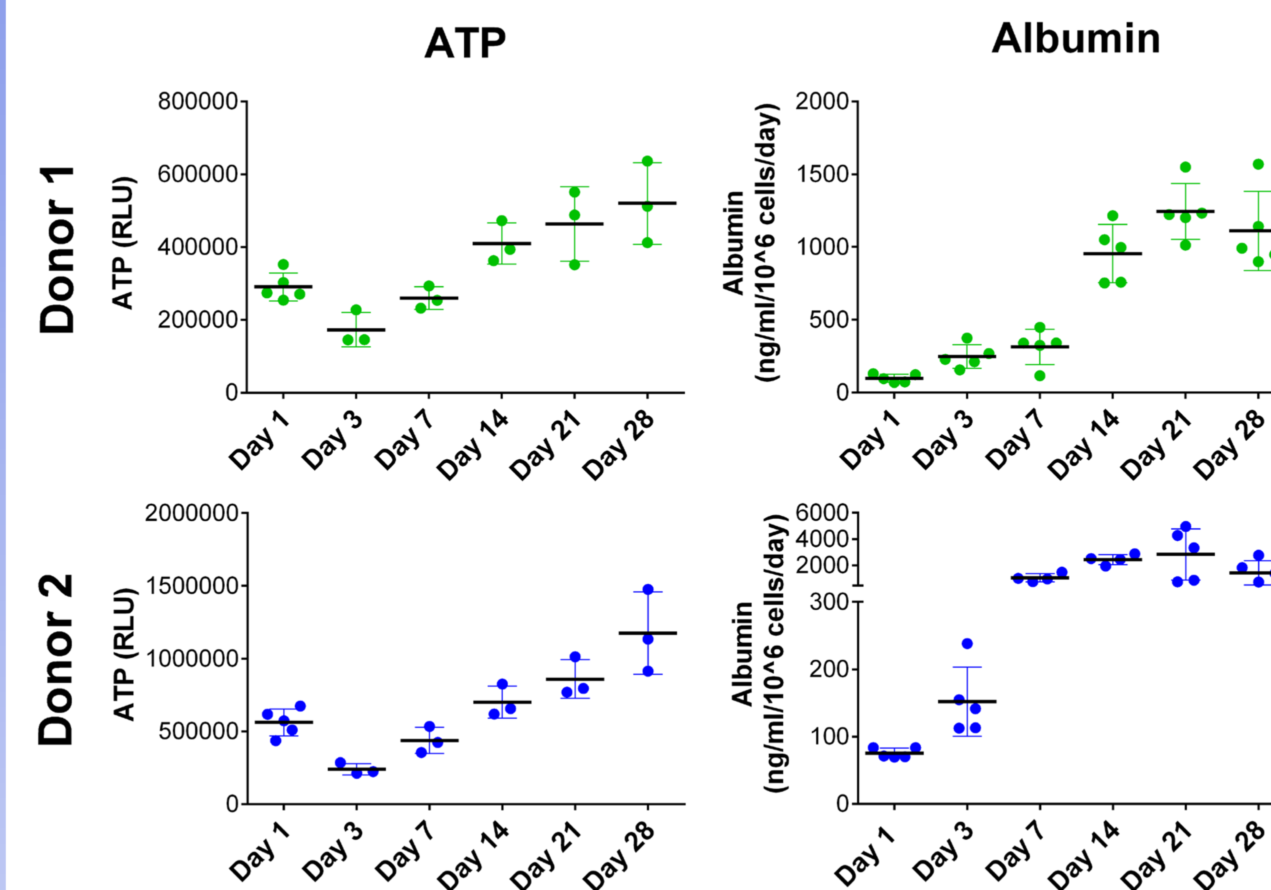

Figure 3: *exVive3D* human liver tissues remain viable and functional for weeks after manufacturing. Tissue viability, as measured by ATP production per tissue using Cell Titer Glo (Promega), stabilizes rapidly after fabrication and increases over time. Production of human albumin, assessed by ELISA, increases after fabrication, stabilizing by Day 14. Data shown is the mean +/- standard deviation for independent tissues generated from two hepatocyte donors.

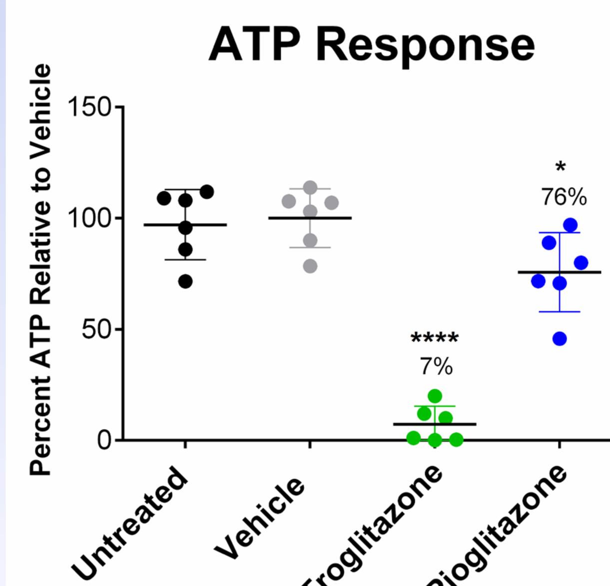

Figure 4: *exVive3D* human liver tissues can differentiate between related toxic and non-toxic compounds. Tissue viability was determined by measurement of ATP (Cell Titer Glo) following 7 days exposure to either vehicle, Troglitazone (100 μM), or Pioglitazone (100 μM). Data shown is the mean +/- standard deviation for independent tissues.

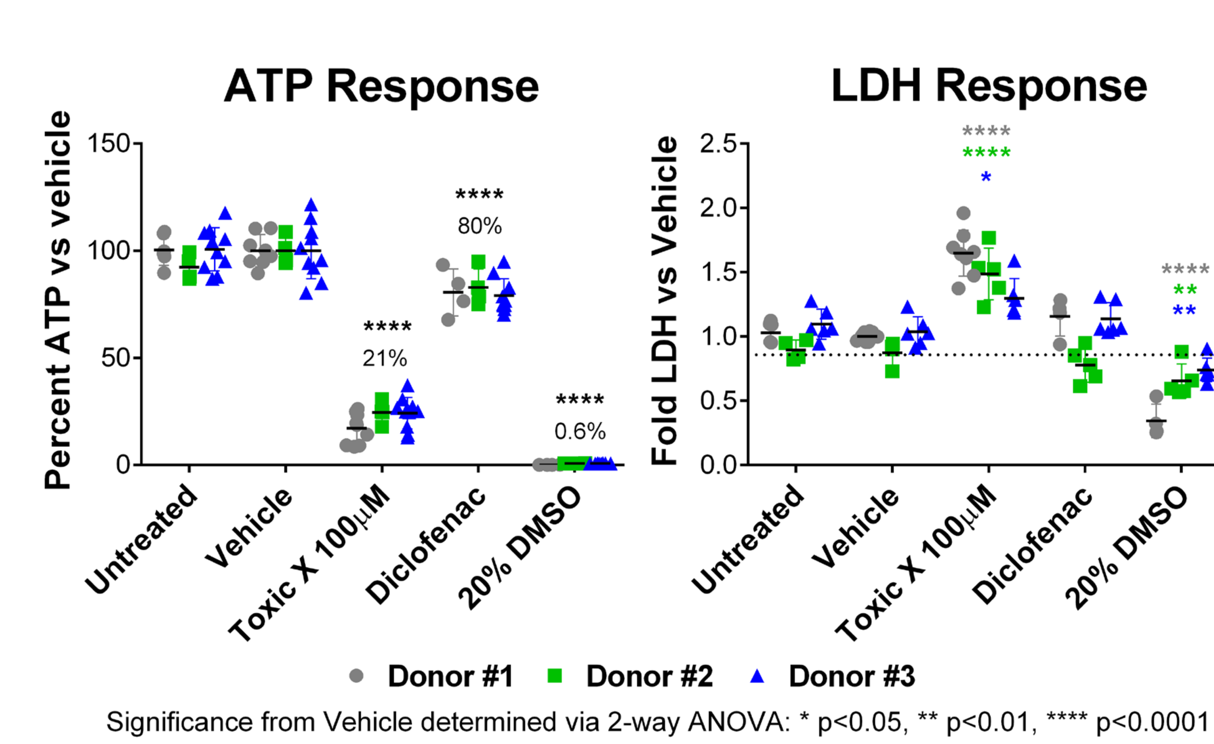

Figure 5: *exVive3D* human liver tissues can effectively model liver injury. *exVive3D* human liver tissues were manufactured from each of (3) human hepatocyte donors and treated with vehicle, a known hepatotoxic compound (Toxic X), Diclofenac, or DMSO. ATP was measured in tissue homogenates (Cell Titer Glo), and LDH in supernatants (Abcam, Inc.). Data shown is the mean +/- standard deviation for independent tissue replicates.

Basal mRNA Expression

Relative Quantity vs GAPDH

CYP2B6 CYP2C9 CYP3A4

Day 3
Day 7
Day 14

Significance determined via 2-way ANOVA with multiple comparisons: ** p<0.01, *** p<0.001, **** p<0.0001

Figure 6: The expression of key CYP450 drug metabolizing enzymes in *exVive3D* liver tissues increases over time. Tissues were maintained in culture for two weeks and basal CYP2B6, CYP2C9, and CYP3A4 relative mRNA expression was measured by qRTPCR, and normalized to GAPDH. Data shown is the mean +/- standard deviation for independent tissues.

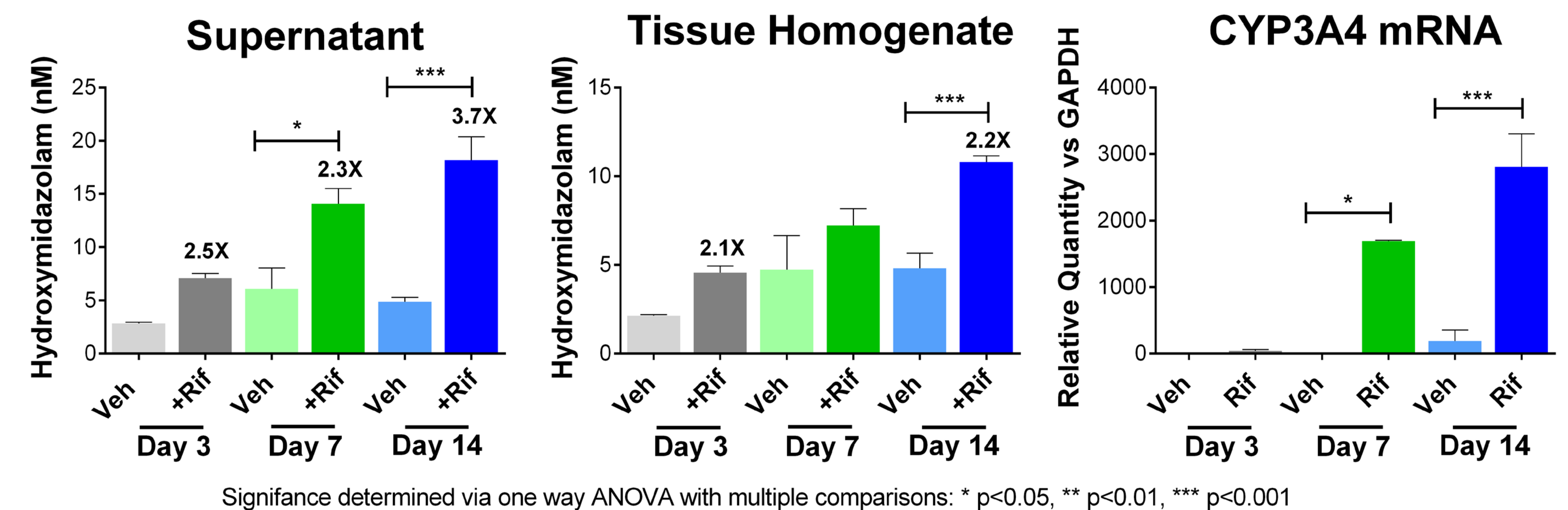

Figure 7: The capacity of *exVive3D* liver tissues for CYP3A4-mediated metabolism of midazolam increased over time, and displayed a 2-3X induction response to Rifampicin. *ExVive3D* human liver tissues were treated for three days +/- Rifampicin (10 μM) starting on the culture day indicated, followed by a 24-hour exposure to Midazolam (10 μM). Levels of 1-hydroxymidazolam were measured in either tissue homogenates or culture media by GC/MS (SciAnalytical Startegies, Inc.). Relative expression of CYP3A4 mRNA was quantified by qRTPCR following induction, and normalized to GAPDH. Data shown is the mean +/- standard deviation for independent tissues.

CONCLUSIONS:

- ***exVive3D* bioprinted human liver tissues retain key aspects of native human liver for up to six weeks:**
  - ➢ **compartmentalized multicellular architecture**
  - ➢ **viability (ATP)**
  - ➢ **expression of liver-specific proteins**
  - ➢ **expression and function of key CYP450 enzymes**
- ***exVive3D* bioprinted human liver tissues respond to prototypical hepatotoxic agents.**

Safe Harbor Statement

Any statements contained in this report and presentations that do not describe historical facts may constitute forward-looking statements as that term is defined in the Private Securities Litigation Reform Act of 1995. Any forward-looking statements contained herein are based on current expectations, but are subject to a number of risks and uncertainties. The factors that could cause actual future results to differ materially from current expectations include, but are not limited to, risks and uncertainties relating to the Company's ability to develop, market and sell products based on its technology; the expected benefits and efficacy of the Company's products and technology; the timing of commercial launch and the market acceptance and potential for the Company's products, and the risks related to the Company's business, research, product development, regulatory approval, marketing and distribution plans and strategies. These and other factors are identified and described in more detail in the Company's filings with the SEC, including its prospectus supplement filed with the SEC on November 27, 2013, its report on Form 10-Q filed February 6, 2014 and its transition report on Form 10-KT filed with the SEC on May 24, 2013 and our other filings with the Securities and Exchange Commission. You should not place undue reliance on these forward-looking statements, which speak only as of the date of this Current Report. These cautionary statements should be considered with any written or oral forward-looking statements that we may issue in the future. Except as required by applicable law, including the securities laws of the United States, we do not intend to update any of the forward-looking statements to conform these statements to reflect actual results, later events or circumstances or to reflect the occurrence of unanticipated events.

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